
# Characterizing the role of p21-Activated Kinase 3 (PAK3) in AP-1-induced transformation

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“The more I study nature, the more I stand amazed at the work of the Creator.”

**-Louis Pasteur**

**CONTENTS**

## ABBREVIATIONS

| | |
|---|---|
| AID | Auto Inhibitory Domain |
| AP-1 | Activating Protein 1 |
| APS | Ammonium Persulphate |
| ATF | Activating Transcription Factor |
| BCA | Bicinchoninic Acid |
| BSA | Bovine Serum Albumin |
| Da | Dalton |
| bp | Base pairs |
| bZIP | Basic Leucine Zipper |
| CA | Constitutively-activated |
| CC | Cervical Cancer |
| CDC42 | Cell Division Control Protein 42 Homolog |
| cDNA | Complementary DNA |
| ChIP | Chromatin Immunoprecipitation |
| cFos | Cellular Fos |
| cJun | Cellular Jun |
| CNS | Central Nervous System |
| $CO_2$ | Carbon dioxide |
| CRE | Cyclic AMP Responsive Element |
| °C | Degrees Celcius |
| DAPI | 4',6-diamidino-2-phenylindole |
| DMEM | Dulbecco's Modified Eagle's Medium |
| DMSO | Dimethyl sulphoxide |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxynucleoside triphosphate |
| dox | Doxycycline |
| EGF | Epidermal Growth Factor |
| EMSA | Electrophoretic Mobility Shift Assay |
| ERK | Extracellular Signal-Regulated Kinase |
| ETS | E twenty six |
| FCS | Fetal Calf Serum |
| GAPDH | Glyceraldehyde 3-Phosphate Dehydrogenase |
| GEF | Guanine Nucleotide-Exchange Factor |
| GFP | Green Fluorescent Protein |
| GTPase | Guanosine Triphosphatase |
| GusB | β-glucuronidase |

| | |
|---|---|
| HB-EGF | Hairpin-Binding Epidermal Growth Factor |
| HOSE | Human Ovarian Surface Epithelium |
| HPV | Human Papilloma Virus |
| hr | Hour |
| IPTG | Isopropyl β-D-1'-thiogalactopyranoside |
| JNK | Jun N-terminal Kinase |
| Kb | Kilobases |
| LB | Luria Broth |
| Luc | Luciferase |
| M | Molar |
| MAP(K) | Mitogen Activated Pathway (Kinase) |
| mg | Milligram |
| min | Minutes |
| ml | Milliliter |
| MTT | 3'-(4',5'-Dimethylthiazol-2'-yl)-2',5'-Diphenyltetrazolium Bromide |
| NFAT | Nuclear Factor of Activate T-cells |
| NF-κB | Nuclear Factor κB |
| ng | nanogram |
| nM | Nanomolar |
| OD | Optical Density |
| PAGE | Polyacrylamide Gel Electrophoresis |
| PAK | p21-Activated Kinase |
| PBD | p21-Binding Domain |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| PEG | Polyethylene Glycol |
| PMSF | Phenylmethylsulfonyl Fluoride |
| Rb | Rentinoblastoma Protein |
| PI3K | Phosphatidylinositol 3-Kinase |
| RIPA buffer | Radioimmunoprecipitation Assay Buffer |
| RNA | Ribonucleic Acid |
| RNAsin | Ribonuclease inhibitor |
| RT | Reverse Transcription |
| shRNA | Short hair-pin RNA |
| siRNA | Small interfering RNA |
| SOC | Serous Ovarian Cancer |
| Sp1 | Stimulating Protein 1 |
| SPARC | Secreted Protein Acidic and Rich in Cysteine |

| | |
|---|---|
| Srx | Sulfiredoxin |
| SSeCKS | Src-Suppressed C Kinase Substrate |
| SV40 | Simian Virus 40 |
| Ta | Annealing Temperature |
| TBST | Tris-Buffered Saline Tween-20 |
| TK | Thymidine Kinase |
| TRE | TPA Responsive Element |
| µl | Microliter |
| UV | Ultraviolet |
| vJun | Viral Jun |
| X-gal | 5’-Bromo-4’-chloro-3’-indolyl β-D-galactopyranoside |

**ABSTRACT**

Previous studies identified p21-Activated Kinase 3 (PAK3), a serine/threonine kinase, as a potential AP-1 target gene. PAK3 has been implicated in a variety of pathological disorders and over-expression of other PAK-family members has been linked to cancer. In this study, we investigated AP-1 regulation of PAK3 expression and the role of PAK3 in cJun/AP-1-associated cellular transformation. Our results showed elevated PAK3 expression at both the mRNA and protein level in cJun-over-expressing Rat1a fibroblasts, as well as in transformed human fibroblasts. Elevated PAK3 protein levels were also seen in cervical, ovarian, oesophageal and breast cancer cells lines, while poor survival tracked with high PAK3 expression in ovarian cancer patient material. Elevated PAK3 levels appear to play no role in the proliferation of transformed or cancerous cells, however appears vital for the transformed morphology and actin distribution. These cytoskeletal changes seem to be the underlying force governing cellular migration, as inhibition of PAK3 significantly reduced the motility of both transformed fibroblasts and cancer cell lines. Our data shows that elevated PAK3 expression in response to AP-1 over-expression is regulated through the transcriptional activation of the PAK3 promoter by AP-1 binding directly to a single site in the promoter. We also show that constitutive activation of PAK3 results in changes in cJun phosphorylation and an increase in AP-1 activity, which can be inhibited by a serine/threonine kinase inhibitor. PAK3 and AP-1 proteins were also shown to directly interact with each other. Our study is a first to describe a role for AP-1 in regulating PAK3 expression, and PAK3 in regulating AP-1 activity, identifying a potential feedback loop in which PAK3 is an AP-1 target required for cytoskeletal reorganization and migration observed in transformed cells.

# CHAPTER 1

# LITERATURE REVIEW

## 1.1. Cancer

Cancer is considered to be a highly complex disease. It is estimated that there are approximately 200 different cancer types or subtypes, with every individual and population bringing additional complexity in the molecular and genetic defects that drive cancer development[1]. According to the World Cancer Report, issued by the International Agency for Research on Cancer (IARC), in 2008 there were an estimated 12.4 million new cases of cancer recorded and 7.6 million deaths caused by cancer globally[2]. This incidence is predicted to increase to 22.2 million in 2030, when 13 million lives are estimated to be claimed by cancer[3] unless more effective interventions and treatments are developed.

## 1.2. The development of cancer

Cancer is a disease that arises when normal cells start "behaving badly", dividing uncontrollably, avoiding or bypassing signals to stop; resulting in the accumulation of a tumour mass. In general, this behaviour often begins with changes or mutations in the genetic material of the cell. Should these mutations continue to accumulate over time, they may result in defects in the normal regulatory circuits that control the proliferation and homeostasis of a cell. These now precancerous or altered cells can proliferate uncontrollably to form lesions. With the accumulation of more genetic alterations, some of the precancerous cells evolve into cancer cells, ultimately forming a tumour. Further mutations in these tumour cells may result in the cancer becoming metastatic, leading to the spreading of the cancer cells and the

development of a secondary tumour (Fig. 1.1). Thus the transformation of a normal cell to a malignant cell is considered to be a complex, multistep and multigene process[4].

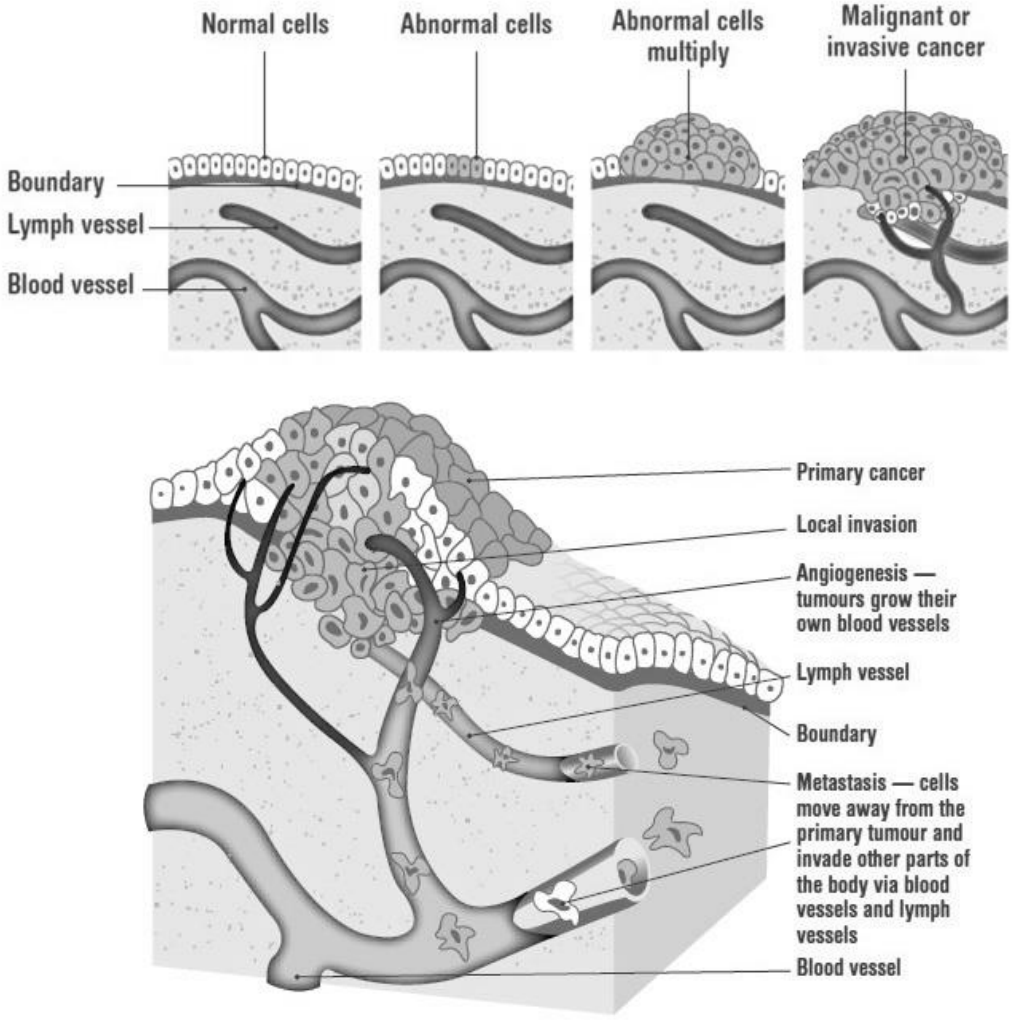


**Figure 1.1: A schematic representation of the progression of a normal cell to a malignant tumour. A**dapted from Understanding cancer – Cancer Council Queensland[235]

Cancer development is associated with dynamic changes in the genome, resulting in a gain of function in genes with the potential to promote cancer development (oncogenes) and a loss of function in genes that prevent the development (tumour suppressors), thereby aiding oncogenesis. Although the exact origin and process of cancer development still remains

unknown, research has resolved underlying patterns and principals common to the development of cancer. Hanahan and Weinberg (2000)[4] outlined six of these traits and later added two more traits necessary for tumour development[5] (Fig. 1.2).

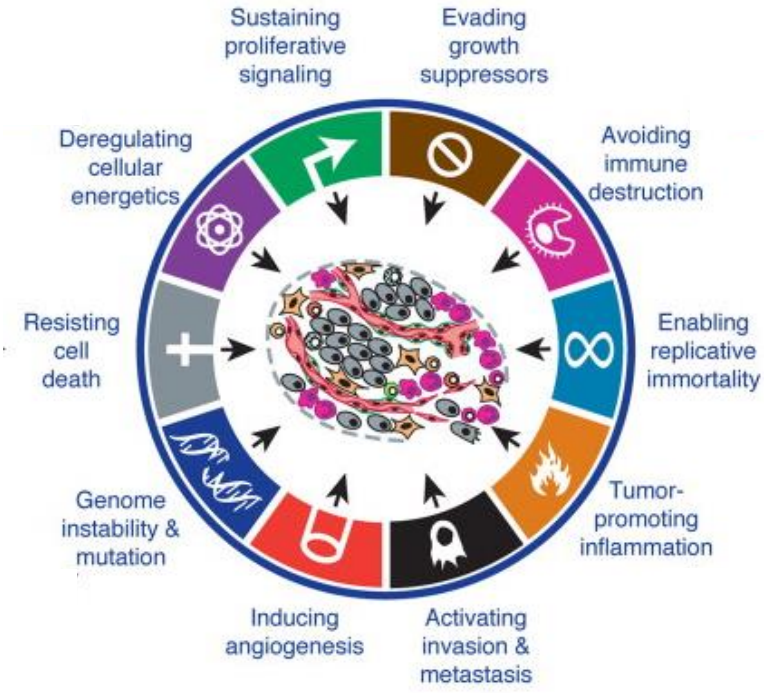


**Figure 1.2: An illustration encompassing the hallmarks of cancer: acquired characteristics and enabling factors necessary for the malignant phenotype.** From Hanahan and Weinberg (2011)[5]

Hanahan and Weinberg propose that there are acquired molecular, biochemical and cellular characteristics that are essential and common to all malignant growths. The order of acquisition of these characteristics may vary significantly across malignancies, both mechanistically and chronologically. These eight hallmarks are:

### *i. Sustaining proliferative signalling*

Malignant cells acquire the ability to be self-sufficient in their growth signals, in this way sustaining their proliferation. Many oncogenes act by mimicking normal growth signalling,

allowing cancer cells to have a remarkably reduced dependence on exogenous growth stimulation.

***ii. Evading growth suppressors***

A normal cell requires multiple antiproliferative signals to maintain cellular homeostasis and quiescence. In order to prosper uncontrollably, malignant cells must acquire the ability to circumvent these powerful antigrowth signals.

***iii. Resisting cell death***

Programmed cell death, apoptosis, is a major source of cellular control, eliminating damaged or unwanted cells, thereby preventing the development of cancer. Malignant cells are required to resist apoptosis and do so by over-riding cellular check-points through the mutation of tumor suppressors.

***iv. Enabling replicative immortality***

Cancer cells must acquire the ability to disrupt the intrinsic programme that limits the multiplication potential of normal cells should the malignant cells expand enough to create a life-threatening tumour.

***v. Inducing angiogenesis***

Like normal cells, cancer cells require nutrients and the ability to eliminate waste. As the size and proliferative ability of the tumour mass increases, this need increases. Cancer cells must therefore acquire the ability to turn on the usually transitory process of new blood vessel growth, angiogenesis.

### *vi. Activating invasion and metastasis*

Often cancer cells will invade their adjacent tissues and spread to a secondary location, escaping their primary site in search of nutrients and space. Secondary tumours, metastases, are the cause of 90% of cancer-related deaths[6], meaning that most tumour masses acquire the ability to lose contact to their primary site, invade and implant at a secondary site successfully.

### *vii. Reprogramming energy metabolism*

Cancer cells rely on rapid and uncontrolled proliferation for the development of a tumour. For this, cancer cells not only rely on sustained proliferation signals and avoidance of apoptotic signals, but also on an adjustment in their energy metabolism in order to fuel their growth. Cancer cells must thus acquire the ability to reprogram their metabolism to meet their energy requirements in the presence of limited oxygen.

### *viii. Evading immune destruction*

Cells and tissues are constantly monitored by the alert immune system, which is able to detect and destroy developing cancer cells. In order to survive, cancer cells have therefore developed the ability to avoid this detection and limit the destruction mediated by the immune response.

In addition to these eight hallmarks, Hanahan and Weinberg (2011)[5] highlighted two enabling characteristics that help promote or aid the development of these hallmarks: genomic instability and mutation, and tumour promoting inflammation. The former suggests that the generation of successive random mutations will promote the acquisition of the above mentioned hallmarks. The second characteristic, tumour promoting inflammation, suggests

that the tumour-associated immune response, originally thought to eradicate the cancer, in fact aids the cells' acquisition of the hallmarks by supplying the tumour with bioactive molecules.

In summary, these hallmarks and enabling factors highlight the complexity of the process required to transform a normal cell to that with a cancerous phenotype. Despite extraordinary progress in the understanding of cancer development over the past 40 years, in many cases and for most forms of cancer, the war has not yet been won[7]. Although the hallmarks of cancer provide a clear model for the characteristics and requirements of cancer development, more recently, there seems to be a return, within research, to again confront the endless complexity of the disease[8].

The process of cancer development, from the initiation and promotion to the progression of tumourigenesis, is throught mainly to be driven by alterations in gene expression, each of which can be classified under one or more hallmark. These changes in gene expression may be a result of various mechanisms including genetic mutations, epigenetic alterations or the dysregulation of transcription factors that control the expression of the altered genes. One such protein, whose deregulation has been seen to individually transform cells in culture, is the transcription factor AP-1[9].

### 1.3. Activating Protein 1 (AP-1): identification and characterization

The Activating Protein 1 (AP-1) is a fundamental transcription factor encompassing a family of transcriptional complexes. This active complex is a dimer of varying combinations of the AP-1

sub-family protein members. Members of this family include several basic leucine zipper (bZIP) domain proteins: the Jun, the Fos, the Activating Transcription Factor (ATF) and the Maf subfamilies[10], where the Jun subunit holds the DNA binding site of the complex[11] (Fig. 1.3). As a result of this, cellular Jun (cJun) has been named as the central component of the AP-1 transcription factor[12].

Jun was originally identified as an oncogene, first from a retrovirus in the form of viral Jun (vJun) and then as cJun, the normal cellular form of the protein. cJun was identified as a transcription factor based on similarity in its DNA-binding domain to an already identified yeast transcription factor, GCN4[13,14]. Subsequently, two other Jun proteins have been identified: JunB and JunD. Despite sharing high sequence and structural homologies, the Jun proteins have distinct expression patterns and effects[15]. Thus, through alternative pairing of these bZIP subunits in the form of Jun:Jun homodimers or Jun:bZIP heterodimers, different classes of AP-1 dimers can be formed[11] (Fig. 1.3).

The major sub-group of the AP-1 complex which most frequently dimerizes with Jun, the Fos group of proteins, is comprised of the following four members: cFos, FosB, Fra1 and Fra2. The two less frequent dimerization partners of Jun are the ATF (AFT2, ATF3/LRF1, B-ATF, JDP1 and JDP2) and Maf (cMaf, MafA, MafB, MafG/F/K and Nr1)[16,17] family of proteins. The number of AP-1 subfamily members and the possible variations of interactions between them, highlight the importance of the regulation required to activate this transcription factor.

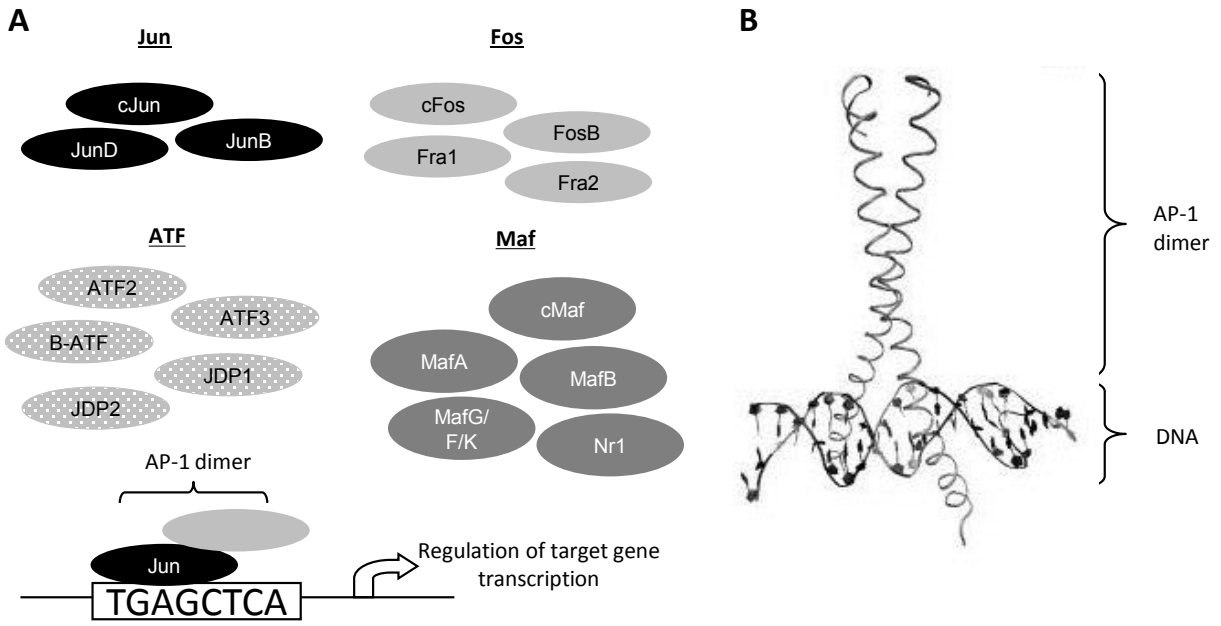


**Figure 1.3: Schematic representation of the AP-1 complex. A:** The AP-1 transcription factor is formed by dimerization of the Jun proteins or heterodimerization of the Jun proteins with the Fos, ATF or Maf subfamilies of proteins. The Jun subunit holds the DNA binding site of the AP-1 transcriptional complex which, once dimerised, binds its consensus sequence in the promoter of its target genes. **B:** The AP-1 subfamily proteins are basic-region leucine zipper (bZIP) proteins and their interaction and interaction with their binding site is shown in this molecular model. Adapted from Chan *et al.* (2012)[236]

## 1.4. Activation of AP-1

Environmental stress, the engagement of cytokine receptors or treatment with growth factors leads to the universal response of a rapid, transient expression and activation of AP-1[18]. AP-1 has therefore been shown to play a significant role in the cellular response to signals generated by radiation, UV, cytokines, growth factors, mitogens and other stresses such as oxidative stress[9,17].

It is the activation of the Mitogen Activated Protein (MAP) kinase pathway, in response to these stresses, that is responsible for the activation of AP-1[18,19]. AP-1 is considered the central transcription factor of the mitogen-activated transduction pathways[17], where the activation

and control of AP-1 requires MAP kinase dependent cJun and cFos transcription and post translational protein modification[20]. Thus, up-stream from AP-1, a stress signal will activate a cascade in the MAP pathway, ultimately resulting in the activation of AP-1 by phosphorylation[20].

### 1.5. Phosphorylation and dimerization of the AP-1 subunits

Transcriptional activation of the AP-1 transcription factor requires the phosphorylation of the appropriate sub-family members, allowing for dimerization and the ability of the AP-1 complex to bind the DNA (Fig. 1.3). The cJun component of the AP-1 complex is phosphorylated on the serine 63 and serine 73 residues of the protein[21,22] . This phosphorylation is performed, most commonly, by the cJun N-terminal kinase (JNK), where JNK 1/2 are thought to be the specific kinases responsible for the phosphorylation[13,23]. However, other kinases, ERK 1/2, have also been seen to phosphorylate these two residues and other AP-1 components[24], suggesting that it is likely that there may be alternative serine kinases - of which there are more than 500 known[25] - capable of performing this function.

Once the subunits are phosphorylated and dimerised, different AP-1 factors bind and regulate different target genes, thus playing distinct biological roles[18]. For example, the cJun/JunB dimer blocks the G1 transition of the cell cycle, whereas the cJun/cFos complex promotes it[11]. As the abundance of the bZIP proteins fluctuate within a cell, the interactions of these dimer-forming-subunits vary[15]. This complex interaction between the subunits as well as the interaction of AP-1 with other transcription factors, such as ETS[26], Smad[27], Sp1[28] NFκB[29] and NFAT[30,31], leads to

the regulation of a large network of AP-1 target genes and biological effects. Although the mechanism of how AP-1 achieves this functional diversity is poorly understood, the advantages thereof allows AP-1 to perform as an "environmental biosensor", regulating the cell physiology in response to many stresses[10].

### 1.6. Transcriptional activation and down-stream effects of AP-1

Once activated, the dimerized AP-1 complex behaves as a classic transcription factor, binding its consensus sites in the promoter of its target genes, either up- or down-regulating their expression. The AP-1 binding site may be either the TPA responsive element (TRE) (5'-TGA G/C TCA-3')[11,32] or the cyclic AMP responsive element (CRE) (5'-TGAGCTCA-3') [33], where different dimers favour different sites. It is thought that the sequence flanking the AP-1 binding site may determine the ability and type of interaction that different Jun proteins have with the site[34]. For example, cJun has been shown to activate promoters containing a single AP-1 binding site, while JunB is reported to require a number of binding sites to regulate activation[35,36].

Through sequence-specificity, regulatory selectivity of the AP-1 binding partners and co-operativity with other transcription factors, AP-1 achieves its down-stream effects through transcriptional activity[33,37]. Depending on the cell type and context, AP-1 has been seen to play a role in cell proliferation, differentiation, survival, inflammation, hypoxia and apoptosis[17,38–40]. All of these biological functions are necessary for neoplastic transformation[17], pointing to a role for AP-1 in oncogenesis.

### 1.7. AP-1: deregulation and role in oncogenesis

The oncogenic signal cascade critically requires the regulation, or more correctly, the deregulation of the AP-1 transcription factor[18]. Both the dominant oncogenes Ha-Ras and Src induce the activation of cJun and cFos[18], where Ras requires AP-1 activity for morphological transformation[41]. AP-1 has been shown to play a pivotal role in tumorigenesis; its activation has been linked to tumor progression and its over-expression has been identified in multiple human cancers such as breast, ovarian, endometrial, colon and lung cancers[42–47].

The central subunit of AP-1, cJun, was originally identified by its ability to transform cells and has since been classified as an oncoprotein[13]. Over-expression of Fos, another major subunit member of the AP-1 complex, has been seen to induce the formation of osteosarcoma in rodents[48,49], where the inhibition of AP-1 blocked the migration, invasion and metastasis of murine osteosarcoma[50].

A number of the AP-1 family members, namely cJun, JunB, JunD and cFos, have been observed to be up-regulated in a genome-wide microarray of human splenic marginal zone lymphomas (SMZL)[51]. Over-expression of cJun and JunB individually caused the transformation of cultured Rat1a fibroblasts[9], and have been shown in Hodgkin's disease[52]. Altered JunB expression was also observed in T-cell lymphomas[35].

Overall, a role for the AP-1 transcription factor has been identified in transformation, angiogenesis, invasive growth, metastasis, deregulated growth and apoptosis [53]. Of the cellular

functions necessary for the development of a clinically significant tumour, metastasis is the most life-threatening[4] and least understood stage of the cancer. The transcriptional activity of AP-1 has been observed to be 3- to 5-fold higher in a highly metastatic version of clonally related murine osteosarcoma[50], and activity of this transcription factor is implicated in the motility and invasion of a number of model systems[54]. Over-expression of cJun also induced invasion in the low metastatic human breast cancer cell line, MCF7[55].

Gene-array analyses, to identify global gene expression patterns[56–58], have shown that cancer cells have different transcriptional profiles compared to normal cells. From these studies, it was noted that there are a number of transcription factors whose activity was specifically required in cancer cells. With variation in transcriptional regulation accounting for much of the diversity of normal and cancer cells, gene expression patterns have become useful tools for identification, classification and treament of tumours. For example, identification of two clinically different subtypes of chronic lymphocytic leukemia (CLL) has now been achieved through a gene expression-based predictor[57]. With transcription factors driving the changes in gene expression responsible and pivotal for the development of cancer, targeting the regulation of such transcription factors could prove a highly effective therapeutic strategy. Furthermore, these transcription factors are both less numerous than their upstream activators and at focal points in deregulated pathways[59], suggesting that these transcription factors would be appropriate targets for cancer therapy. However, how to target or inhibit these identified transcription factors remains the question.

### 1.8. Targeting AP-1 for cancer therapy

Transcription factors have traditionally been thought to be too difficult to target as the object of a therapeutic drug, as they have extensive protein-protein interfaces and a general lack of hydrophobic pockets. However, the hormone/steroid receptor class of transcription factors has the ability to bind natural small-molecules inhibitors, allowing them to be specifically targeted. For example, extensive work has been done utilizing tamoxifen, a drug that targets the estrogen receptors in breast cancer cells[60] and bicalutamide, an androgen receptor inhibitor exploited in prostate cancer[61]. For many years, the steroid receptors were the only class of transcription factors to successfully be targeted; however with the development of technologies and a better understanding of transcription factor functioning, various advances have been made.

cJun has been implicated as a promising therapeutic target for vascular remodelling, acute inflammation, rheumatoid arthritis and cancer[62]. Protective immunity suppressors, thiobarbituates, have been shown to inhibit AP-1 dependent gene expression and AP-1 complex formation at clinically relevant doses in T lymphocytes[63]. Additionally, a fruit extract from Phyllanthus emblica (P.emblica), also known as the Indian Gooseberry, has been discovered to have the ability to inhibit AP-1 in cervical cancer cells[64]. This inhibition of AP-1 is thought to occur via the AP-1 subunit members: cJun, JunB, JunD and cFos. Although the mechanism of action and the active ingredient in the plant extract is unknown, this seems to be the first time a specific inhibitor to AP-1 has been identified.

Despite the discovery of potential AP-1 inhibitors, the efficacy of targeting AP-1 must be questioned as AP-1 has been implicated in chemotherapeutic drug resistance in a number of cell lines[65,66] and, despite being up-regulated in a number of cancers, it also plays a vital role in the normal functioning of cells. It is thought that there is a subset of AP-1's target genes that maintain and regulate its oncogenic function and phenotype[13]. These targets, individually or in combination, may be more effective and druggable targets than AP-1 itself.

### 1.9. AP-1 target genes associated with cellular transformation

To date, four AP-1 responsive genes have been identified to play a partial role in AP-1 associated transformation: a hairpin-binding epidermal growth factor (HB-EGF)[67], a scaffold protein, SSeCKS[68], an extra cellular matrix protein, SPARC[55], and an enzyme that regulates reactive oxygen species, Sulfiredoxin (Srx)[69]. Although research has revealed information about the signalling cascades that activate AP-1 and the functional role this expression plays in cancer, many of the down-stream effectors of this transcription factor complex remain to be identified.

### 1.10. Identification of PAK3 as an AP-1 target gene

In order to identify AP-1 target genes necessary for cellular transformation, Kinoshita *et al.* (2003)[70] established a system of Rat1a fibroblasts with inducible cJun expression. Induction of cJun/AP-1 in this model system was sufficient to produce two transformed phenotypes: a distinct transformed cell morphology and anchorage-independent growth. Analysis using cDNA microarrays on mRNA, extracted from control and doxycycline-induced cJun/AP-1 expressing cells, identified a number of genes with differential expression upon cJun induction.

These genes showed involvement in the cell cycle progression, metabolism, growth, signal transduction, adhesion and architecture of the membrane and extracellular matrix[9] - all vital processes involved in transformation. PAK3, a signal transduction molecule which was a previously undescribed AP-1 target gene, was found to be up-regulated in response to cJun/AP-1 over-expression.

### 1.11. Identification of the PAK proteins

The first PAK protein, PAK1, was discovered in a study aimed at identifying proteins that interact with the G protein, Rac[71]. It was noted that the kinase activity of PAK1 could be stimulated by binding activated (GTP-bound) Rac, as well as CDC42[71]. Rac and CDC42 are both members of the Rho GTPase family of proteins, a group of the Ras superfamily of small GTPases. The Rho family of proteins have been shown to regulate cellular actin dynamics, cell movement, organelle development and various other cellular functions[72,73]. These small GTPases are given their name based on their low molecular weight of approximately 21 kDa. In turn, the protein identified and shown to hold kinase activity in response to Rac and CDC42, was named p21-Activated Kinase (PAK).

After the discovery of the first PAK, three other PAK-related mammalian proteins capable of binding CDC42 and Rac were identified, leading to the nomenclature PAK1 (PAKα), PAK2 (PAKγ), PAK3 (PAKβ) and PAK4[74,75]. A yeast two-hybrid screen addressing proteins that interact with the androgen receptor identified the next PAK member, PAK6[76]. The final member of the

family, PAK5, had been predicted from a sequence data base, and was identified using a degenerate primer against PAK4[77]. Since PAK1 was the first of the family to be identified, it is the most characterized and studied of the PAK proteins. PAK1 and PAK3 share the most sequence similarity, suggesting similar functional roles for these two PAK proteins.

### 1.12. The PAK family of proteins

The PAK family is divided into two subsets, Group 1 (PAK1, PAK2 and PAK3) and Group 2 (PAK4, PAK5 and PAK6), where the members are grouped according to the similarity of their domain-architecture[78] (Fig. 1.4). All six members share a similar N-terminal regulatory domain containing the Rac/CDC42 binding site, known as the p21 binding domain (PBD), while only the Group I PAKs share the inclusion of an auto-inhibitory domain alongside or within this PBD. The C-terminal contains the more highly conserved catalytic/kinase domain. This domain shares

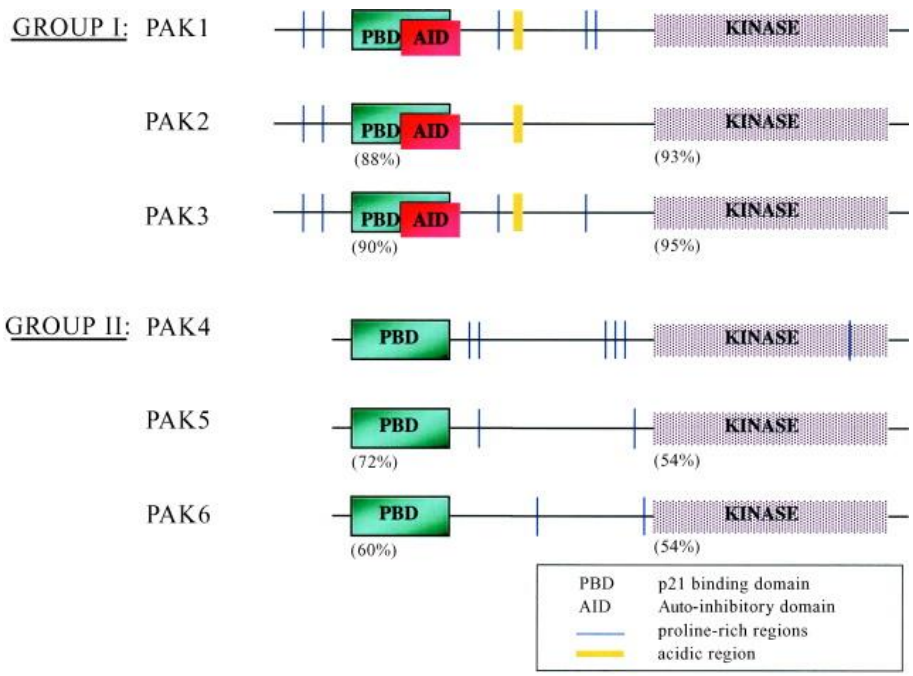


**Figure 1.4: The architecture of the PAK family of proteins.** This diagram is a structural comparison of the PAK proteins showing the defining features of the two groups and the degree of domain conservation as percentages, relative to PAK1 for Group I, and PAK4 for Group II. The PBD and kinase domains are highly conserved across all the PAK proteins, where only the Group I proteins possess an AID. (The diagram is not drawn to scale.) From Jaffer and Chernoff (2002)[78]

93% homology between the members of the Group I, while only 75% between the members of Group II[78]. These differences between the individual PAK protein domains are thought to allow for the different regulations and functions of this family of proteins.

### 1.13. Activation of the PAK family of proteins

As evident from their different architectures, the Group I and Group II PAK proteins are differentially activated. Although both groups interact with the small GTPases, members of Group I require this binding for activation[71], while Group II PAK proteins are activated independently and their interaction with the small GTPases determines their sub-cellular localization, rather than their activity[79]. PAK proteins, which are mostly cytoplasmic, are recruited to the plasma membrane to be activated.

The Group I proteins are activated when bound by the activated form of CDC42 or Rac[71,80]. The activation of CDC42 and Rac occurs as a result of the upstream activation of guanine nucleotide-exchange factors (GEFs)[71]. These GEFs stimulate the GTP loading of the small GTPases in response to activation of membrane-bound growth factor receptors, integrin cell adhesion complexes and G-coupled protein receptors.

The binding of the GTPases to the PAK protein occurs in the N-terminal regulatory region, which in the Group I of PAK proteins, is thought to hold two regions: the p21-binding domain (also known as the G-protein binding domain) and the auto-inhibitory domain (or negative regulatory region)[81]. Investigations done on PAK1 have revealed that this auto-inhibitory domain is

thought to contain three internal regions, namely: the dimerization domain, the inhibitory switch domain and the kinase inhibitory segment – which interacts with and inhibits the activity of the kinase domain[82,83]. Despite this work being done on PAK1, it is thought that the following model applies to all Group I PAK proteins since they have such similar sequence identity. The PAK proteins are thought to form dimers with one another, aligning the auto-inhibitory domain of one monomer with the kinase domain of the other, ensuring both proteins are held in an inactive state[82,84]. Once bound by an activated GTPase, a conformational change in the inhibitory domain results in the dissociation of the dimer and phosphorylation and activation of the PAK monomers[83,84]. Once activated, it is through sustained binding from the GTPases, as well as an interaction with adaptor proteins, such as NCK, GRB2a and PIX, that the subcellular localization of the activated Group I PAK proteins is determined[85–87]. These adaptors interact with the PAK proteins through the proline-rich regions of the protein: NCK interacts with the first proline-rich sequence and PIX through an atypical proline-rich sequence[80,88,89].

The activation of PAKs by the GTPases results in the perpetuation of the activating signal from the upstream pathways to achieve the downstream effects. Although other kinases such as MEKK1/4 have been shown to interact with Rac and CDC42[90], the Group I family of PAKs were the first identified targets, and are known to be the primary direct effectors, of the small Rho-GTPases[71].

### 1.14. GTPase-independent PAK activation

Although PAK proteins are generally thought to be activated by the binding of the small GTPases, there are alternative mechanisms through which PAK proteins have been seen to be activated. For example, PAK proteins have been activated through a direct interaction with PI3K[91], while AKT has been shown to activate the PAK proteins indirectly[92]. PAK1 has been shown to be dose-dependently activated by an interaction with sphingosine and other lipids[93]. Phosphorylation by PDK1 has been shown to activate PAK1[94], while phosphorylation by PKA inactivates PAK protein[95]. The hyper-phosphorylation, by a complex of cyclin-dependent kinase 5 (CDK5) and p35, results in the down-regulation of PAK1 activity[96].

Other reports of PAK activation include the activation of PAK2 by caspase-mediated proteolysis of the N-terminal portion of the PAK2 protein[97–99]; an activation which appears to be specific to PAK2. It has been shown that the inclusion of a membrane-targeting CAAX box in the PAK protein sequence resulted in activation of PAK, even in mutants unable to bind G-proteins such as Rac and CDC42[93].

### 1.15. Down-stream effects of the PAK proteins

The specific upstream signals dictate the degree of activation and, along with binding partners, localization of the PAK proteins. Following activation, these serine/threonine kinases then activate their down-stream effectors by phosphorylating them at specific serine or threonine residues, where they notably favour serine residues over threonines as substrates [100].

The main activators of the PAK proteins, the Rho proteins, have distinct functions; different from those of the Ras superfamily to which they belong. These functions include cytoskeletal reorganization and motility, as well as the production of reactive oxygen species (ROS)[101,102]. Specifically, CDC42 controls the formation of filopodia and Rac1 the formation of lamellipodia. The PAK proteins were first discovered to be involved in cytoskeletal dynamics and actin depolarization[103,104]. Subsequently, PAKs have been found to be involved in many other downstream functions. These down-stream effects include growth-factor and steroid-receptor signalling, signal transduction, survival, mitosis, energy homeostasis, gene transcription, and cytoskeletal reorganization to effect cell morphology and motility[78,105]. It has been hypothesised that there are two important end points to PAK signalling: nuclear events that influence gene expression and cytoskeletal events that impact upon cellular dynamics[106].

Although the family of PAK proteins have a defined role, loss of function experiments of individual PAK proteins have shown their non-complementary functions[107,108]. The members of the Group I PAK family show a large discrepancy in their involvement in biological systems. PAK1 is thought to rescue the adverse effects of autism, but advance the severity of Huntington's disease[109,110]. PAK2, activated by cleaved caspase-3, controls morphological changes associated with apoptosis[105]. PAK3 is seen to be mutated in certain X-linked mental retardation syndromes and other deficiencies associated with learning problems[111,112].

Such varied functions suggest an interesting regulation of PAK proteins. Also, the diversity of inputs and outputs of PAK proteins suggest that PAKs play an important role in the key

functioning of cells. This would then further suggest a potential role for the PAK proteins in pathological disorders and oncogenesis.

### 1.16. PAK proteins in transformation and cancer

The first evidence to implicate PAK proteins in cancer came when the exogenously-introduced p21-binding domain (PBD) of PAK1 inhibited Ras- and Rac-induced transformation in Rat1 fibroblasts[113]. The kinase activity of PAK1 was shown to be needed for Ras-induced transformation[114], placing PAK1 signalling at a central point in the transformation signals arising from the small GTPases. PAK1 expression was seen to be up-regulated in ovarian, breast, bladder and lymph cancers [113,115], and has been shown to stimulate cell migration[79].

PAK1 and PAK2 have both been linked to neurofibromatosis type 2 (NF2), where these PAK proteins have been shown to block the activity of the NF2 tumour suppressor gene, Merlin[116,117]. Specifically, PAK2 negatively regulates the expression of the oncogenic protein, Myc[118], and has been seen to play a dual role in apoptosis, regulating it both positively and negatively. Elevated PAK2 expression has also been seen in prostate, ovarian and breast cancer[115,119,120].

Members of the Group II family of PAK proteins have also been implicated in cancer. PAK4 is elevated in 78% of all tumour cell lines and promotes focus formation in cells[121]. It is localized to a region on chromosome 19 that is also commonly amplified in some colon and ovarian tumours[121]. In pancreatic cancer, the PAK4 locus itself is amplified[122]. Activated PAK4 results in

anchorage-independent growth in both human[121] and mouse cells[121], specifically that associated with oncogenic transformation by Rho-GTPases[123]. Inactivation of PAK4, like PAK1, blocks Ras-induced transformation[121].

Another member of the PAK Group II family, PAK6, has been shown to bind the androgen and oestrogen receptor-α in breast cancer, and, like PAK1, renders cells insensitive to treatments such as tamoxifen[124,125]. Like the other PAKs, PAK6 expression has also been seen to be elevated in prostate cancer[115].

As signal transduction molecules, the PAK proteins exist as intermediates between many signalling pathways and are thought to propagate their upstream effector signals, amplifying oncogenic signals. One such signal may be the activation of the transcription factor AP-1. With the Group I PAK proteins being the primary effectors of the small GTPases, the PAK proteins are placed up-stream of the activation of AP-1. AP-1 is primarily activated through phosphorylation by JNK[13,23], which in turn is activated through the MAP Kinase pathway[126]. The PAK proteins are thought to often pass their activating signal on through the MAP Kinase pathway to the JNK proteins[71]. Although PAK1 and PAK2 have not been identified as AP-1 target genes in the microarray analysis performed by Kinoshita *et al*. (2003)[70], PAK3 was. Taken together, this information suggests that there may be signaling feedback loop between AP-1 and PAK3 which may amplify the oncogenic signal.

### 1.17. PAK3: an introduction

PAK3, also known as βPAK, was first identified and purified from brain tissue as a 65-68 kDa protein [127]. Evidently, the function of PAK3 lies specifically within the embryonic development and physiology of the neurons and the associated cognitive processes[71,128]. PAK3 is the only PAK Group I protein whose expression is thought to be relatively restricted to the neurons [71,129,130]. This is in contrast to PAK1, which is seen to be highly expressed in the nervous system and also in other tissues[129].

Unlike other PAK proteins, PAK3 is the only family member to be chromosomally conserved across species to the X-chromosome. Along with its role in neuronal development, its gene location has resulted in PAK3 being highly implicated in pathological disorders, such as certain X-linked mental retardation syndromes[111] and learning problems associated with synaptic plasticity[112].

The PAK3 gene itself is also unique compared to the other PAK proteins, as it contains two alternatively spliced exons, generating four splice variants[130]. These different variants may potentially account for the variation in the PAK3 protein size (see Fig. 1.5.) The basic PAK3 variant, PAKa, does not contain either of the alternative exons, while the addition of these exons, forms variants PAK3b, PAK3c and PAK3cb, resulting in the constitutive activation of the protein[130]. This is because these exons are located within the C-terminal regulatory domain of the protein, specifically within the region that the auto-inhibitory domain (AID) and the p21-binding domain (PBD) overlap. The inclusion of either or both of these exons disrupt the

function of this domain, activating the protein and preventing these variants from dimerizing and inhibiting the function of the other PAK3a variants[131]. The addition of these exons resulted in a marked decrease in the interaction of PAK3 with the active GTPases, compared to PAK3a. The alternatively spliced exons are highly conserved during evolution, suggesting that these variants aid and highlight the specific and unique functions of PAK3[130]. Thus, with PAK3 having a link to other pathological disorders, and with the strong association of other PAK family members with transformation, it is likely that PAK3 too has a role in the oncogenic phenotype.

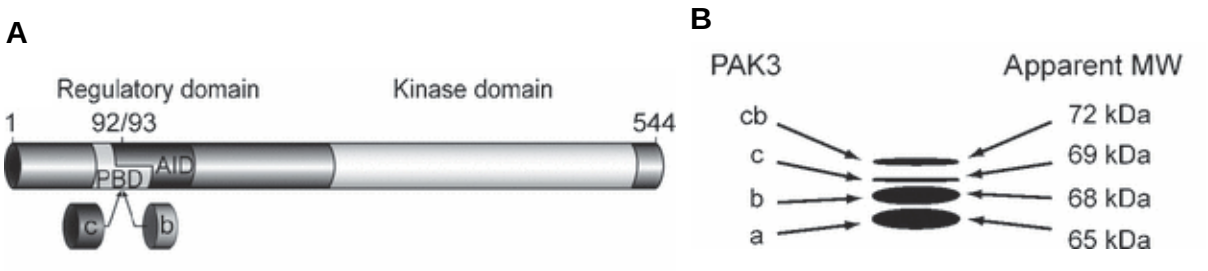


**Figure 1.5: Schematic representation of the alternatively spliced exons and variants of PAK3. A:** PAK3 protein structure and localization of the c and b inserts in the overlapping PBD (p21-binding domain)/AID (auto-inhibitory domain). **B:** Schematic representation of the sizes and relative amount of each splice variant deduced from immunoprecipitated and western blotted protein extract from mouse brain extracts. Adapted from Kreis P et al. (2008)[130]

## 1.18. A potential role for PAK3 in oncogenesis

PAK3, sharing great structural similarity with PAK1, has, like PAK1, been shown to promote anchorage-independent growth through the phosphorylation of Raf1[105]. Similarly, altered PAK3 expression has been implicated in changes in adhesion molecules at focal adhesions sites and alterations in cell morphology[130].

Mutations in PAK3 have been detected in lung adenocarcinomas[132], while PAK3 expression is elevated in cervical cancer HeLa cells[133]. Cancer-specific methylation of PAK3 was found at a high frequency in oesophageal, lung, cervical, bladder and head and neck cancer, while methylation of the PAK3 gene was only occasionally methylated in normal tissues[134].

A dual knock-out study of p53 with PAK3 in cervical carcinomas outlined a synthetic lethal interaction[133], suggesting a link between PAK3 and cancer survival. PAK3 has also been shown to promote cell cycle exit and differentiation of beta-cells of the pancreas[135]. Similarly, changes in PAK3 expression were related to migration of neurons[136].

The above mentioned evidence, along with the PAK family link to cancer and the unique characteristics of PAK3, suggest that PAK3 may indeed play a role in oncogenesis. Little is known regarding the mechanisms that regulate PAK3 expression and its expression patterns in cancer cells of different tissue origins. Having been identified as an AP-1 target gene, PAK3 may be a potential candidate as one of the AP-1 targets required for maintenance of the transformed phenotype.

### 1.19. Significance

The transcription factor, AP-1, plays a significant role in the oncogenesis of many different types of cancers. It mediates this role by regulating the expression of its down-stream target genes that are ultimately responsible for the oncogenic phenotype. PAK3 has been identified as a potential AP-1 target gene, with the potential to play a role in the transformed phenotype

associated with AP-1 oncogenesis. As a serine/threonine kinase, PAK3 may not only be up-stream of the activation of AP-1 but, since kinases have proven to be a promising class of targets for cancer therapy[137], may prove a promising target.

### 1.20. Project aims

This study aimed to investigate PAK3 as an AP-1 target gene involved in cancer development to further understand the down-stream oncogenic signalling mechanism of AP-1 and to characterize the potential role of PAK3 in cancer. The objectives for this investigation were:

i) To characterize the cJun/AP-1 regulation of PAK3 expression, by determining

(A) the mRNA and protein expression of PAK3 in response to cJun over-expression, and

(B) the effect of cJun/AP-1 on the PAK3 promoter;

ii) To determine the level of PAK3 expression in transformed and cancer cells and the biological effect of PAK3 inhibition in these cells;

iii) To determine the effect of constitutively-activated PAK3 on AP-1 and the potential of a regulatory feedback-loop.

# CHAPTER 2

# INVESTIGATION OF THE TRANSCRIPTIONAL REGULATION OF AP-1 ON PAK3 IN A RAT MODEL SYSTEM

## 2.1. INTRODUCTION

AP-1 (Activating Protein 1) is a term used to refer to a class of dimeric transcription factors, comprised of the Jun, Fos, ATF and Maf subunit members, where the Jun subunits hold the transcription factor DNA-binding domain[10,11]. The expression and activation of the AP-1 transcription factor occurs as a rapid and transient response to environmental stress, the engagement of cytokine receptors or the treatment with growth factors[17]. Once dimierized, and thus activated, the AP-1 complex binds one of two consensus motifs in the promoters of its target genes: the TPA responsive element (TRE) (5'-TGA G/C TCA-3') or the cyclic AMP responsive element (CRE) (5'-TGAGCTCA-3')[11,32,33].

Each AP-1 dimer is thought to have specific functions, and thus, as the expression of the subunit proteins fluctuate within a cell, the interactions of these dimer-forming units vary, achieving functional diversity[15]. Additionally, the sequence flanking the AP-1 binding site may determine the ability and type of interaction that different dimers have with the site[24]. For example, cJun dimers activate promoters containing a single AP-1 binding site, while JunB dimers require a number of binding sites to regulate activation[34,35]. Through the interaction with other transcription factors such as Smad, NFκB and NFAT[27,29–31], the regulation that AP-1 exerts on its

targets is further reaching and more specified. The biological functions of AP-1 are thus diverse, ranging from cell proliferation and differentiation to apoptosis[9,17].

With a role in such fundamental cellular functions, deregulation of AP-1 has been highly implicated in cancer. The oncogenic signal cascade critically requires the expression of the AP-1 transcription factor[18] and hence AP-1 over-expression has been seen in a number of cancers[42–45]. Constitutive over-expression of cJun/AP-1 has been shown to cause cellular transformation within Rat1a cells[138] and this transformed phenotype includes changes in cell proliferation, morphology and the induction of anchorage-independent growth[9]. Over all, AP-1's role in oncogenesis is diverse, with the deregulated activity of AP-1 being linked to transformation, angiogenesis, invasive growth, metastasis, deregulated growth and apoptosis[53].

Although there is substantial evidence for AP-1's role within oncogenesis, very little is known about its target genes that are essential for these processes[10]. It is thought there is a subset of its target genes whose deregulation is vital for the maintenance of the transformed phenotype[13]. Microarray analysis performed in order to identify cJun/AP-1 target genes involved in the transformed phenotype, identified PAK3, amongst others, to be elevated in response to cJun/AP-1 over expression[70].

PAK3, a serine/threonine kinase signal transduction molecule, is a member of the p21-Activated Kinase (PAK) family of proteins. This family of proteins is mainly thought to be activated by the small GTPases, CDC42 and RAC[71], however certain other proteins have been shown to activate

PAKs via specific protein-protein interactions or enhancer sites in the PAK promoter[93]. With the preliminary findings of the microarray identifying PAK3 as an AP-1 target gene, the aim of this chapter was confirm and characterize the regulation of AP-1 on PAK3. This was done by determining the effect of cJun/AP-1 over-expression on the expression levels of PAK3 and on the PAK3 promoter.

## 2.2. RESULTS

To investigate the role of AP-1 on PAK3 expression, a rat cell culture model was used to over express cJun/AP-1. In this chapter, the rat model system will first be described, followed by assaying the effect of cJun/AP-1 over-expression on endogenous PAK3 expression and PAK3 promoter activation.

### 2.2.1. A rat model system for cJun/AP-1 over-expression

In order to specifically assay the effects of cJun/AP-1 over-expression, an inducible rat model system was utilized. The experimental system was a rat fibroblast cell line, Rat1a-J4, stably transfected with a doxycyline-inducible cellular Jun (cJun) construct (Fig. 2.1A). cJun may be controllably expressed within these cells allowing for its over-expression and the resultant dimerization of Jun:Jun homodimers or Jun:bZIP heterodimers within the cell[11], where the increased Jun subunit levels drives the formation of dimers equilibrium towards Jun homodimers[55].

As the control for the non-specific effects of doxycycline treatment, a rat fibroblast cell line, Rat1a-GFP, stably transfected with a green fluorescent protein (GFP) construct under doxycycline control was used (Fig. 2.1B).

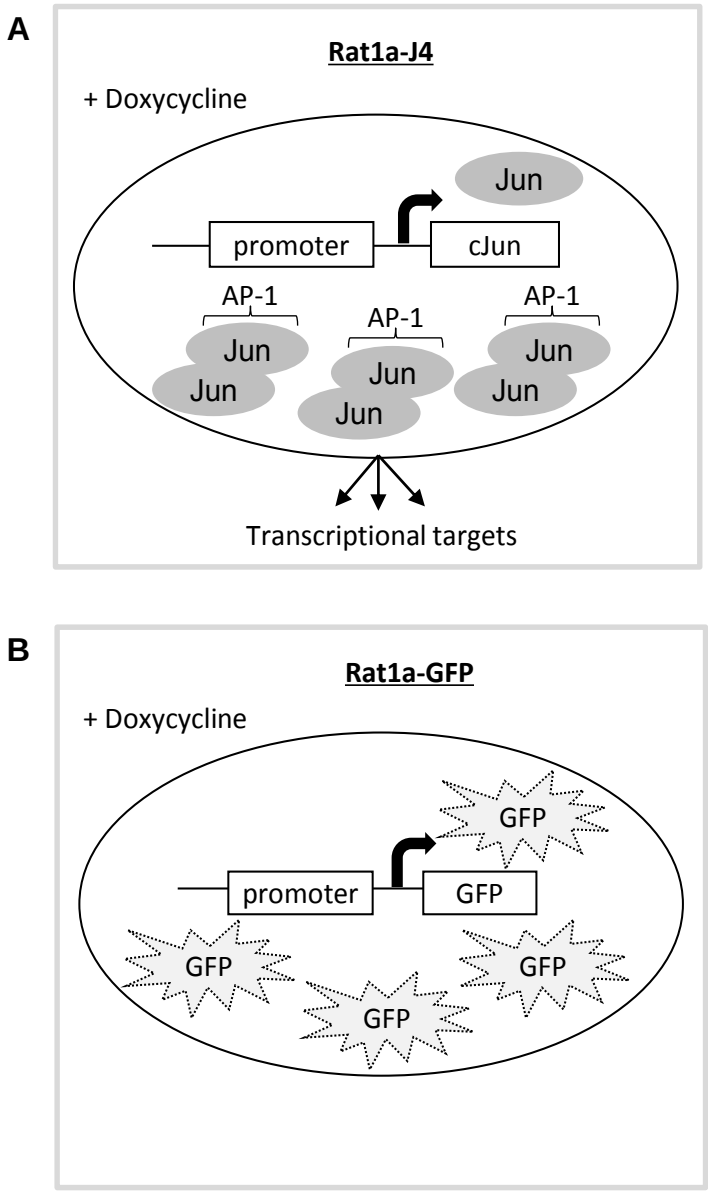


**Figure 2.1: Schematic representation of the inducible rat model system used for AP-1 over-expression.** **A:** The experimental cell line, Rat1a-J4. Parental rat fibroblasts, Rat1a cells, were stably transfected with a docycyline inducible cJun construct allowing for the over-expression of the cJun:cJun homodimer AP-1 complex,. This cell line was used to measure the downstream effects of cJun/AP-1 over-expression. **B:** The control cell line, Rat1a-GFP. Parental rat fibroblasts, Rat1a cells, were stably transfected with a docycyline inducible GFP construct. This cell line was used as a control for the negative effects of doxycycline. Both cell lines were maintained in media containing blasticydin as a selection marker for the stable transfection.

### 2.2.2. Over-expression of cJun/AP-1 results in increased PAK3 expression

Microarray expression analysis identified PAK3 mRNA, amongst others targets, to be up-regulated in cJun/AP-1 over-expressing cells[70]. To independently confirm PAK3 mRNA expression in response to cJun/AP-1 induction, quantitative real-time RT-PCR was used. RNA samples from control rat fibroblasts, Rat1a-GFP, and the experimental rat fibroblasts, Rat1a-J4, grown in both anchorage-dependent and anchorage-independent conditions were subjected to RT-PCR analysis using primers designed against rat PAK3. PAK3 mRNA expression was normalized to the expression of *glyceraldehyde 3-phosphate dehydrogenase* (GAPDH), in order to correct for the potential difference in RNA loaded. This house-keeping gene could be used as a normalizer as it displayed minimal variation between samples, as both the control and experimental cell line were derived from the same parental cell line, Rat1a.

After ensuring the amplification of a single product by melt curve analysis, data from the real-time RT-PCR was analysed using the comparative threshold cycle ($C_T$) method[139] to calculate the expression of PAK3 mRNA between samples. This result showed that PAK3 mRNA levels are significantly up-regulated in Rat1a-J4 cells in response to doxycycline-induction of cJun/AP-1 over-expression, compared to the un-induced Rat1a-J4 cells and the control cells, in both anchorage-dependent (Fig. 2.2A) and anchorage-independent (Fig. 2.2B) growth conditions.

Similarly, western blot analysis performed on protein harvested from cultured Rat1a-J4 cells with or without doxycycline showed a significant increase in PAK3 protein levels in response to

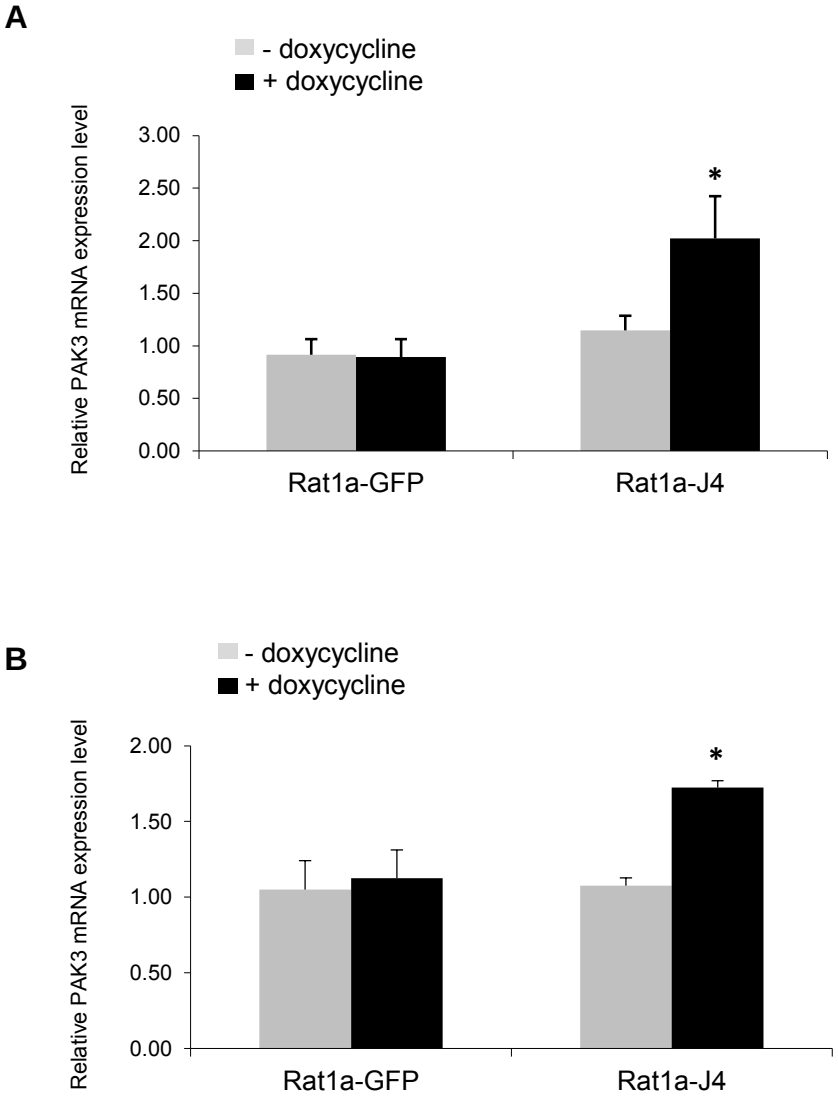


**Figure 2.2: Over-expression of cJun/AP-1 results in increased PAK3 mRNA expression. A & B:** Real time RT-PCR analyses of PAK3 mRNA expression in control cells, Rat1a-GFP, and cJun-inducible cells, Rat1a-J4, grown in the absence and presence of doxycycline for 48 hours. Cells were grown in anchorage-dependant **(A)** and anchorage-independent **(B)** conditions. Results are the mean $\pm$ S.E. of three independent experiments. (*p≤0.05)

cJun/AP-1 over expression in both anchorage-dependent (Fig. 2.3A) and anchorage-independent (Fig. 2.3B) growth conditions. The protein expression levels of the other Group 1 PAK proteins, PAK1 and PAK2, remained relatively unchanged in response to the cJun/AP-1 induction (Fig. 2.3C). Thus, the elevated PAK3 expression in response to cJun/AP-1 induction suggests that PAK3 is specifically an AP-1 responsive gene.

These results show that PAK3 mRNA (Fig. 2.2) and protein levels (Fig.2.3) were found to be significantly elevated in response to cJun/AP-1 over-expression in both growth conditions. The subsequent question was whether the increased PAK3 expression in response to cJun over-expression was as a result of transcriptional or post-transcriptional events. To address this, the PAK3 promoter was cloned into a reporter vector system.

### 2.2.3. Cloning of the PAK3 promoter

To investigate whether the regulation of AP-1 on PAK3 was at the transcriptional level, a region of the rat PAK3 promoter was cloned. Approximately 2.5 Kb of the regulatory region upstream of the PAK3 transcriptional start site, together with approximately 100 bp of the 5' untranslated region downstream of the transcriptional start site was amplified using PCR. This (-2436/+149) region of the PAK3 promoter was then cloned into the shuttle vector, pGEM-T Easy and the resultant plasmid was sequence confirmed. Utilizing the inserted restriction enzyme sites incorporated into the amplification primers, MluI and XhoI, the PAK3 promoter region was cloned into the luciferase reporter vector, $pGL_3$-Basic (Fig. 2.4A). The final vector was mapped using the incorporated MluI and XhoI restriction enzyme sites, as well as an additional set of

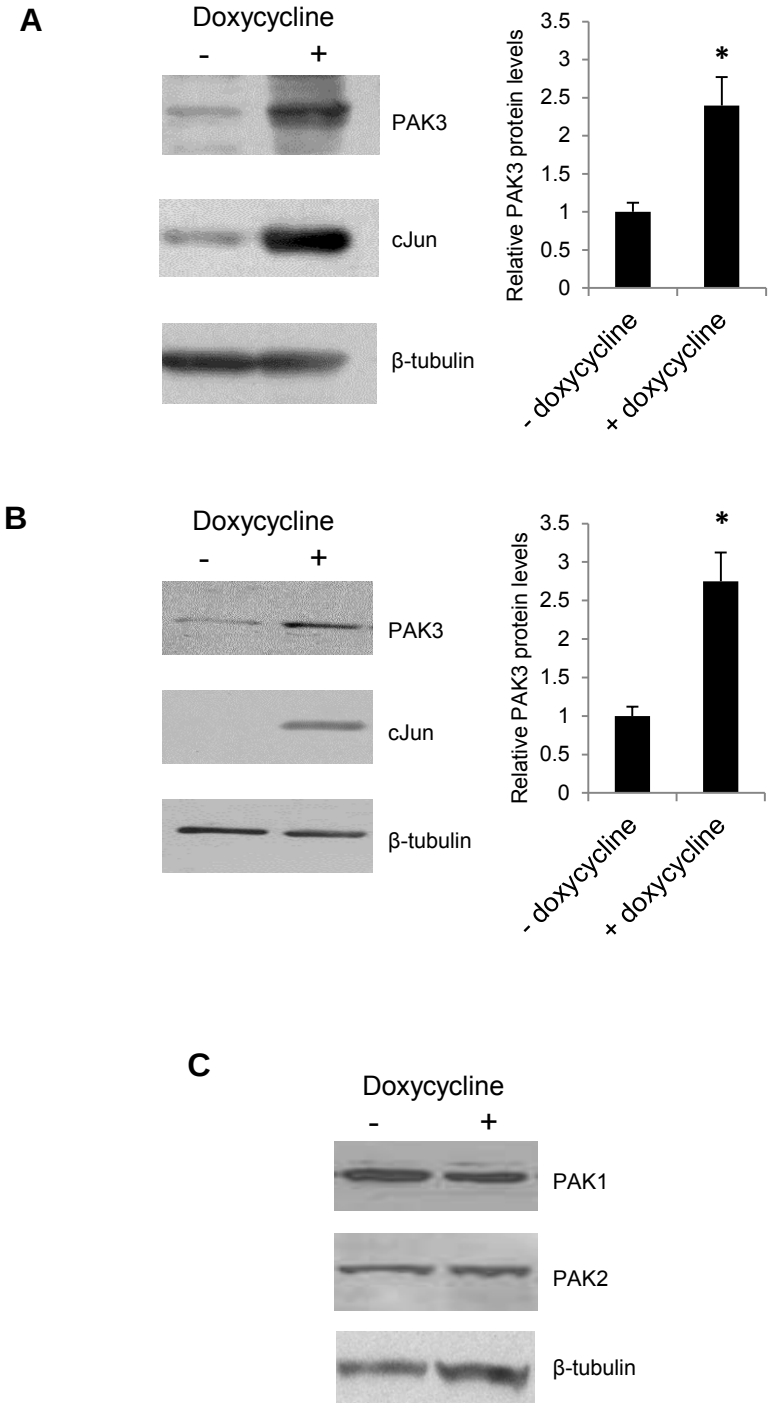


**Figure 2.3: Over-expression of cJun/AP-1 results in increased PAK3 protein expression. A & B:** Western blot analysis showing PAK3 and cJun protein expression levels in Rat1a-J4 cells grown with or without doxycycline for 72 hours in anchorage-dependent **(A)** and anchorage-independent **(B)** growth conditions. Bar graphs represent PAK3 protein levels, relative to the β-tubulin levels in each sample, ± S.E. over three independent experiments. (*p≤0.05) **C:** Western blot analysis showing levels of other Group I PAK proteins, PAK1 and PAK2, in response to doxycycline induction in anchorage-independently grown inducible Rat1a cells.

sites that cut within the insert, EcoRV and HindIII (Fig. 2.4B), confirming the incorporation of the (-2436/+149) region of the PAK3 promoter into the vector.

### 2.2.4. cJun/AP-1 over-expression activates PAK3 promoter activity

Having successfully cloned the (-2436/+149) PAK3 promoter region, we next investigated the effect of cJun/AP-1 over-expression on the activity of the PAK3 promoter. The $pGL_3$-Basic-pPAK3 (-2436/+149) plasmid, alongside the empty vector, $pGL_3$-Basic, was transiently transfected into Rat1a-GFP and Rat1a-J4 cell lines in the presence and absence of cJun/AP-1 over-expression and the luciferase activity measured using the dual-luciferase™ reporter assay (Promega). As an internal control for transfection efficiency, the pRL-TK vector, which contains the thymidine kinase promoter upstream of a Renilla luciferase gene, was simultaneous transfected into the cell lines. Renilla luciferase levels for each sample were used to normalize the luciferase driven by the PAK3 promoter region.

An increase in the luciferase activity was seen in all cells transfected with the $pGL_3$-Basic-pPAK3 (-2436/+149) plasmid compared to the empty vector, $pGL_3$-Basic, indicating that the (-2436/+149) region of the PAK3 gene contains significant promoter activity (Fig. 2.5A). A further significant increase in luciferase activity was detected in Rat1a-J4 cells, transfected with the PAK3 promoter plasmid, in the presence of doxycycline, compared to un-induced cells. This effect was not observed in Rat1a-GFP control cells.

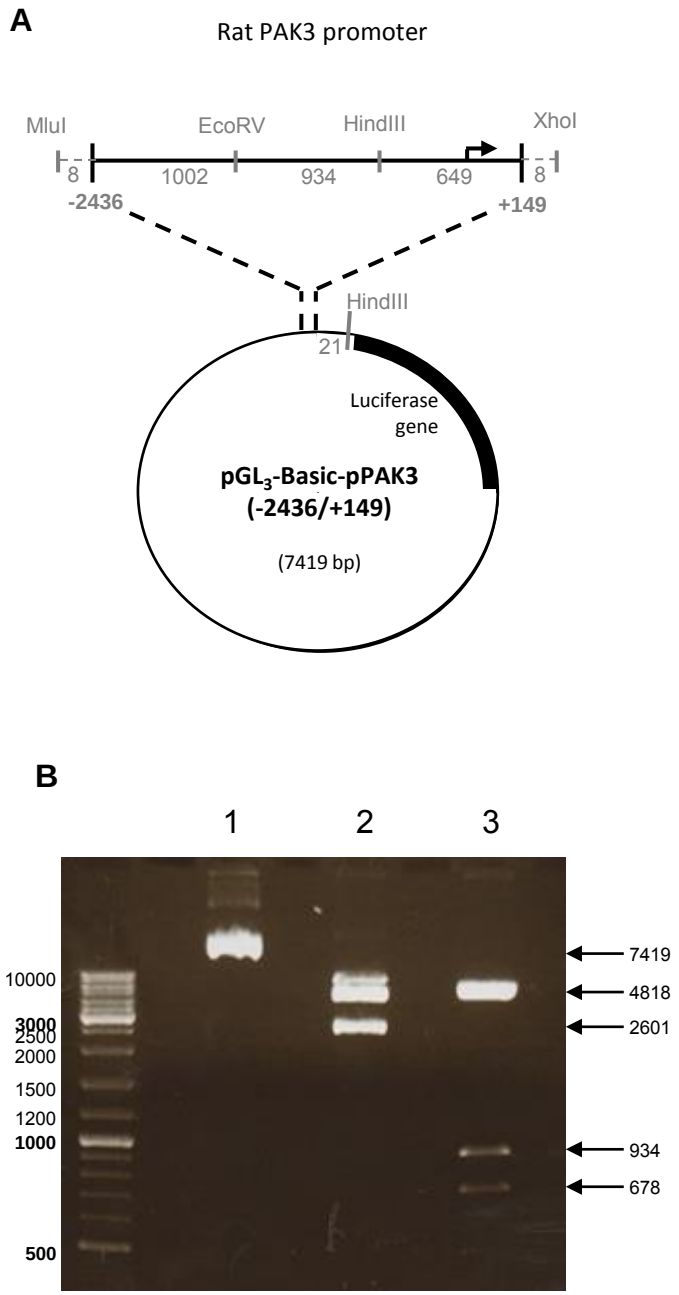


**Figure 2.4: Cloning of the PAK3 rat promoter into $pGL_3$-Basic. A:** Schematic representation of the (-2436/+149) region of the PAK3 promoter in $pGL_3$-Basic. The promoter fragment was inserted upstream of a luciferase reporter gene using restriction enzymes MluI and XhoI. EcoRV and HindIII sites within the insert, along with the HindIII site in the multiple cloning site, were used for mapping. The number of base pairs between each of these sites are represented as grey numbers. **B:** Restriction enzyme digestion of the (-2436/+149) PAK3 region cloned into $pGL_3$-Basic. Digestion products were electrophoresed on a 1% agarose gel. Lane 1, uncut plasmid (7419 bp). Lane 2, MluI/XhoI digestion to release the insert (2601 bp) from $pGL_3$-Basic (4818 bp) . Lane 3, EcoRV/HindIII digestion to yeild 3 bands (4787 bp, 934 bp and 678 bp).

As an alternative method of AP-1 over-expression, transient transfection of pCMV-cJun into Rat1a parental cells also showed a significant, 2.3 fold increase, in the (-2436/+149) PAK3 promoter activity (Fig. 2.5B). A reporter construct containing four wildtype AP-1 binding sites upstream of the luciferase gene, 4XAP-1-Luc (WT), was used as a positive control of AP-1 activity in pCMV-cJun transfected cells (Fig. 2.5B).

These results show transcriptional activation of the PAK3 gene in the presence of cJun/AP-1 over-expression.

### 2.2.5. Putative AP-1 binding sites are present in the (-2436/+149) PAK3 promoter region

Having shown that cJun/AP-1 over-expression plays a role in activating the (-2436/+149) region of the PAK3 promoter, bioinformatic analysis was done to identify potential AP-1 binding sites within this region. Using three software programs, MatInspector[140], ConReal (Conserved Regulatory elements anchored alignment algorithm)[141] and MATCH, several putative AP-1 binding elements, on both the plus and minus strand, were identified (Fig. 2.6).

### 2.2.6. Promoter deletion and mutation analyses identifies a single, controlling AP-1 binding site in the (-2436/+149) PAK3 promoter region

In order to address important control regions and/or putative AP-1 binding sites that may be involved in the activation of the PAK3 (-2436/+149) promoter, a series of deletion constructs were made. These promoter deletion constructs were generated using restriction enzyme digestion to remove small fragments from the 5' end of the PAK3 (-2436/+149) promoter

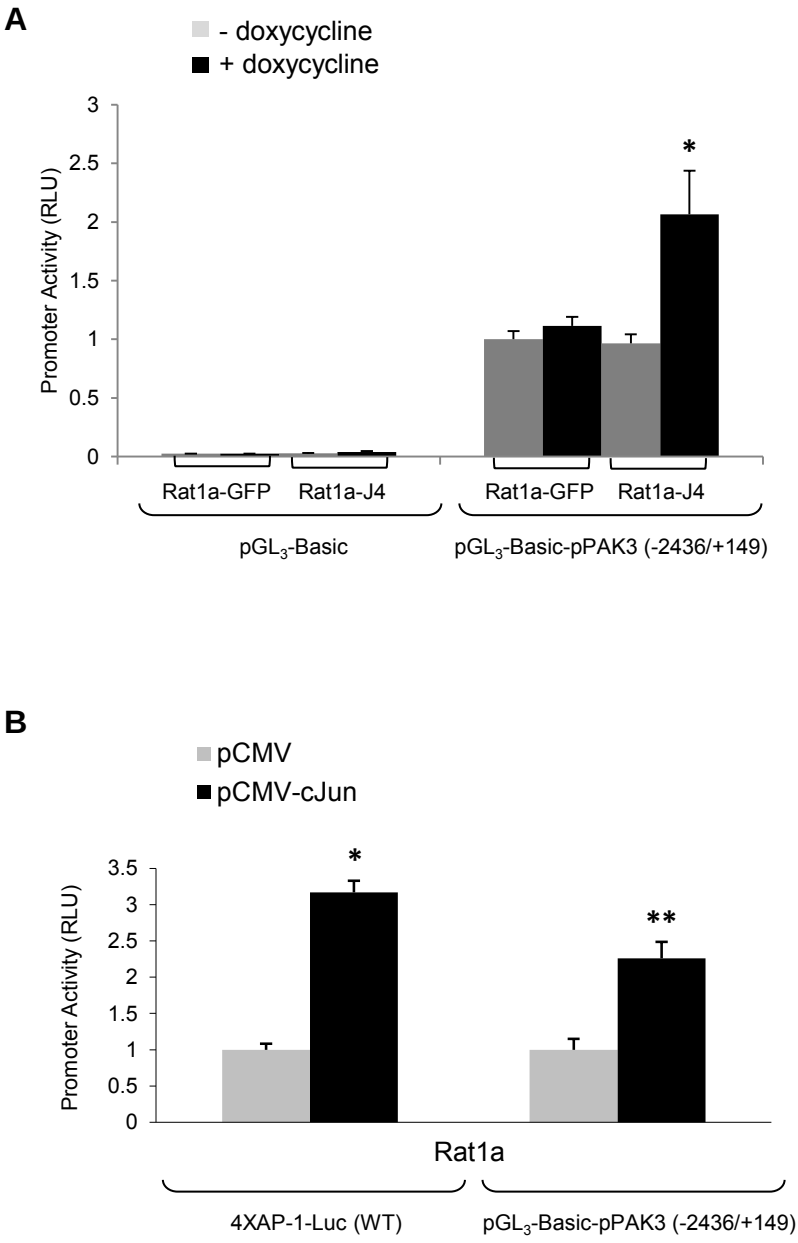


**Figure 2.5: cJun/AP-1 over-expression causes increased PAK3 promoter activity. A:** Luciferase promoter reporter assays performed in Rat1a-GFP and Rat1a-J4 cells transiently transfected with the PAK3 promoter construct containing vector, $pGL_3$-Basic-pPAK3 (-2436/+149) and the empty vector, $pGL_3$-Basic. Cells were grown in the absence and presence of doxycycline for 48 hours to induce cJun/AP-1 overexpression. **B:** Luciferase promoter reporter assays performed in parental rat fibroblasts, Rat1a, transfected with either pCMV-cJun or empty the vector, pCMV, with the PAK3 promoter construct containing vector, $pGL_3$-Basic-pPAK3 (-2436/+149). A plasmid containing four wildtype AP-1 binding sites, 4XAP-1-Luc, was used to show cJun activation. For all promoter assays, TK-Renilla-Luciferase was used as an internal control for transfection efficiency and luciferase activity was expressed relative to Renilla luciferase in each sample. Results show the mean $\pm$ S.E. of experiments performed in triplicate and repeated three times. (*$p \leq 0.05$ and **$p \leq 0.01$).


**Figure 2.6: Putative AP-1 binding sites present in the (-2500/+981) region of the PAK3 promoter.** Putative AP-1 transcription factor binding sites were identified using Conreal, MATCH and MatInspector software programmes and these regions are represented on the above sequence of the (-2500/+981) PAK3 promoter.

region. The cloned MluI site on the 5' end of the fragment was used in conjunction with various restriction enzyme sites present within the promoter fragment. After re-ligation of the cut plasmid, eliminating a small region, a series of truncated PAK3 promoter regions had been generated: namely the (-2329/+149), (-684/+149) and (-179/+149) regions. All constructs were confirmed by restriction enzyme digestion analysis (data not shown).

When transiently transfected into Rat1a-J4 cells, all deletion constructs showed increased promoter activity in response to doxycycline-induced cJun/AP-1 expression (Fig. 2.7). The smallest of the deletion constructs, the (-179/+149) region, despite containing only a single putative AP-1 binding site at position (+52/+60), retained the significant response to cJun/AP-1 seen in all the PAK3 promoter constructs. This AP-1 binding site and the region surrounding it was found to be conserved in other species, such as human, mouse and dog. The site itself, with the sequence of TGACGTCA, is a classic CRE consensus site.

In order to identify the role that the AP-1 binding site, at position (+52/+60), has on PAK3 promoter activity, site-directed mutagenesis was employed to disrupt the site in the (-179/+149) PAK3 promoter construct. The site was mutated to ACGCGTTT (where the underlined regions highlight the mutated bases), creating a unique restriction enzyme site which was used to confirm the presence of the mutation through restriction digestion analysis (data not shown). The mutation was carefully introduced so as to not disrupt any other transcription factor binding sites or add any new ones.

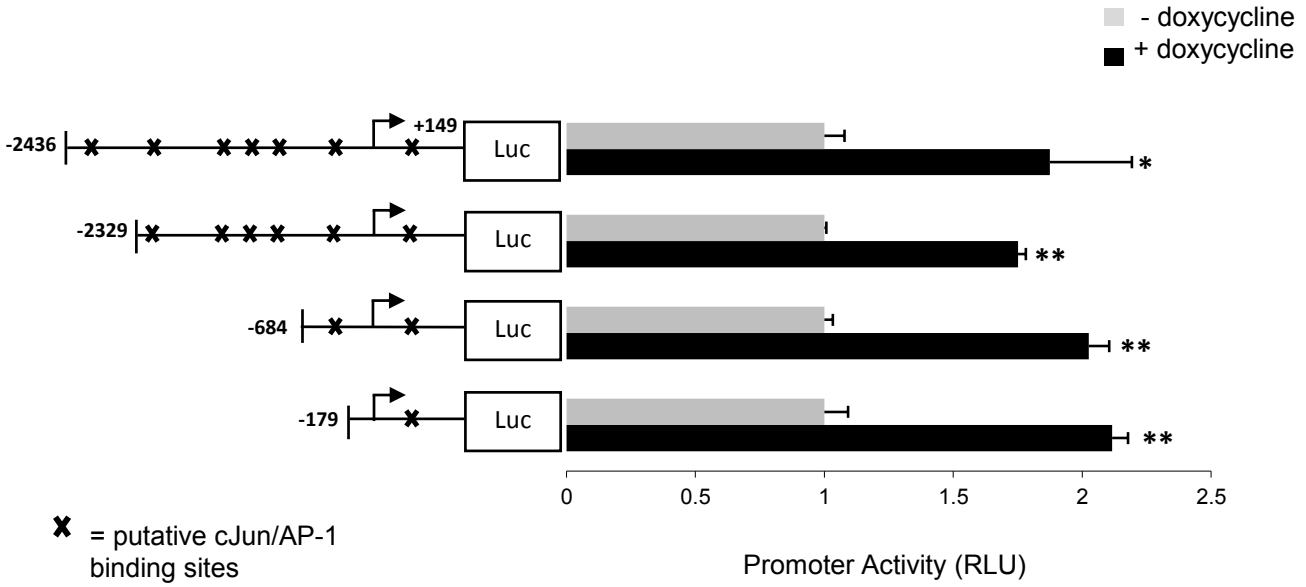


**Figure 2.7: cJun/AP-1 over-expression causes increased promoter activity in all PAK3 promoter deletion constructs.** Luciferase reporter promoter assays for deletion constructs of the PAK3 promoter, namely (-2436/+149), (-2329/+149), (-684/+149) and (-179/+149) promoter regions, in the presence and absence of doxycycline-induced cJun/AP-1 expression. For all promoter assays, TK-Renilla-Luciferase was used as an internal control for transfection efficiency and luciferase activity was expressed relative to Renilla luciferase for each sample. Nucleotides are numbered relative to the transcription start site, represented with by an arrow. Results show the mean $\pm$ S.E. of experiment performed in triplicate and repeated three times. (*$p \leq 0.05$ and **$p \leq 0.01$).

This mutated construct, along with the wildtype (-179/+149) construct, was transfected into Rat1a-J4 cells in the absence and presence of doxycycline-induction of cJun/AP-1 over-expression. The mutated AP-1 binding site at (+52/+60) resulted in a significant loss of the increased PAK3 promoter activity seen in the presence of cJun/AP-1 over-expression (Fig. 2.8A). This indicates that the AP-1 binding site at position (+52/+60) is necessary for the increased PAK3 promoter activity, and hence the elevated transcriptional expression of the PAK3 gene in response to the doxycycline induction of cJun/AP-1.

To ascertain whether this increased promoter activity, via the (+52/+60) site and seen in response to doxycycline treatment, is specifically due to increased cJun/AP-1 levels, siRNA was used to transiently knock-down cJun protein levels. This was done with the wildtype (+179/+149) promoter construct in control and doxycycline-treated cells. Inhibition of cJun did not affect the basal activity of the un-induced (+179/+149) promoter construct, while the knock-down of cJun affected the increased promoter activity seen in doxycycline-induced cJun over-expressing cells (Fig. 2.8B). This result provides further evidence that cJun is required for PAK3 promoter activation.

### 2.2.7. AP-1 binds directly to the (+52/+60) PAK3 promoter region

As PAK3 promoter deletion and mutation assays suggested that AP-1 regulation on the PAK3 promoter occurs in the (+52/+60) region, we used *in vitro* and *in vivo* binding assays to investigate whether AP-1 is directly regulating PAK3 expression through binding this region of the PAK3 promoter.

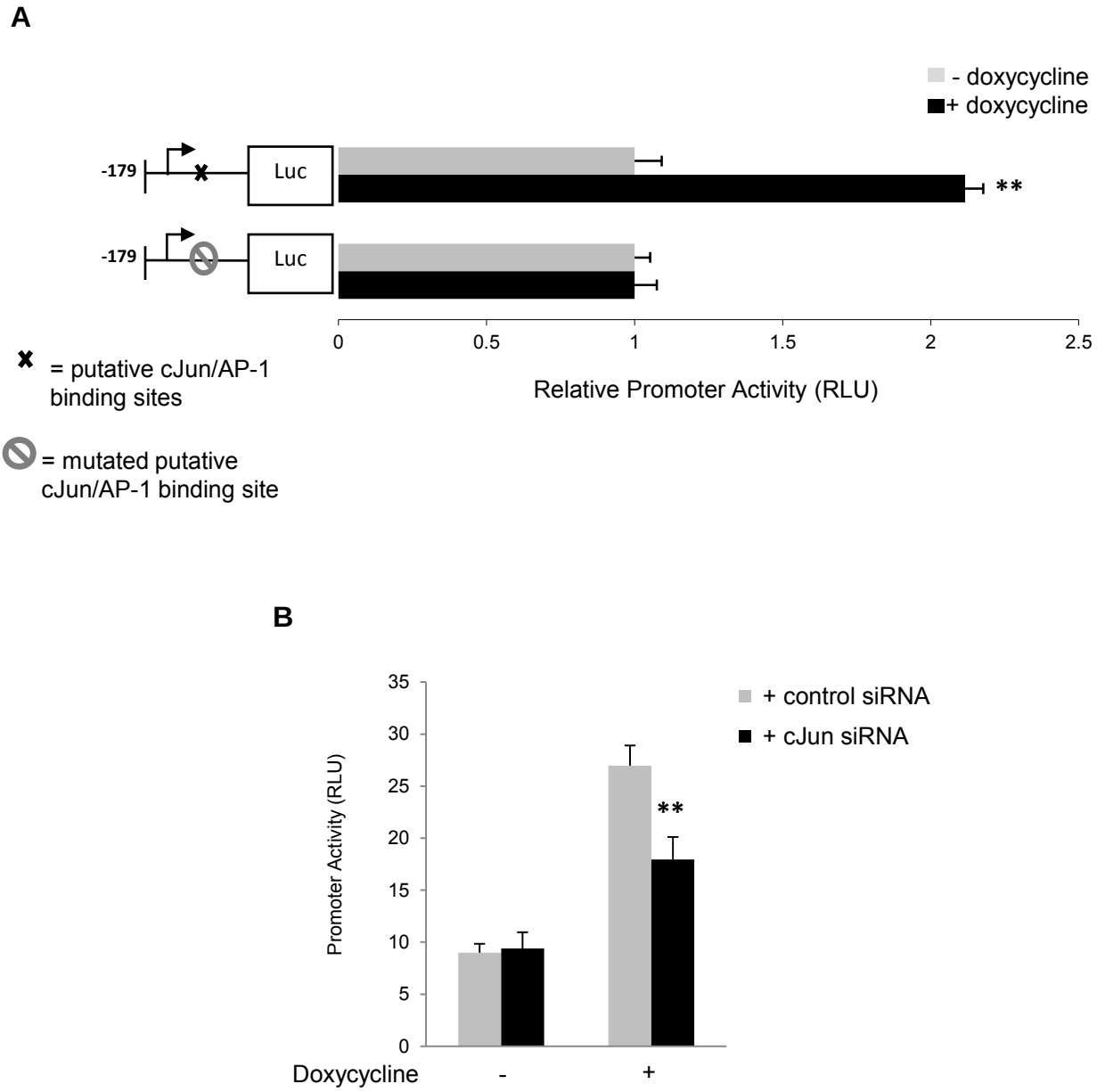


**Figure 2.8: cJun/AP-1 over-expression causes increased PAK3 promoter activity via a putative AP-1 binding site at position (+52/+60). A:** Luciferase reporter promoter assays with a vector containing the (-179/+149) PAK3 promoter region with a wildtype or mutated cJun/AP-1 binding site at position (+52/+60), in the presence and absence of doxycycline-induced cJun/AP-1 expression. A significant loss of promoter activity, in the presence of doxycycline, was seen when the (+52/+60) cJun/AP-1 site was mutated in this construct. **B:** Luciferase reporter assays, using the (-179/+149) PAK3 promoter construct with a wildtype (+52/+60) cJun/AP-1 binding site, showing the effect of transient cJun inhibition on PAK3 promoter activity in the absence and presence of doxycycline induced cJun/AP-1 over-expression. Results show the mean $\pm$ S.E. of experiments performed in triplicate and repeated three times. (*$p \leq 0.05$ and **$p \leq 0.01$).

An Electrophoretic Mobility Shift Assay (EMSA) was performed using a radiolabeled double-stranded oligomer spanning the (+52/+60) region of the PAK3 promoter (Fig. 2.9, panel 1, lane 1– free probe). This *in vitro* binding assay showed the presence of a DNA-protein complex in the presence of protein cell lysate from Rat1a-J4 cells (panel 1, lane 2). In the presence of protein lysate from Rat1a-J4 cells treated with doxycycline, the intensity of the complex appeared increased (panel 1, lane 3).

Further investigation of this DNA-protein complex in the presence of doxycycline induction of cJun/AP-1 over-expression is shown in Fig 2.9 panel 2. The presence of this DNA-protein complex was competed for with the addition of excess unlabelled wildtype (+52/+60) oligomers (lane 5), while excess mutated unlabelled (+52/+60) oligomers did not outcompete the DNA-protein complex (lane 6).

In order to determine which protein forms part of the DNA-protein complex, supershift analysis with various Jun antibodies was done. Two cJun antibodies were used: one targeting the DNA binding domain and the other the N-terminus of the cJun protein. JunB and JunD antibodies were also used. The addition of the antibody targeting the cJun DNA binding domain showed no super-shifted complex (lane 7), presumably because the DNA binding domain epitope is hidden in the DNA-protein complex. However, the addition of a cJun antibody targeting the N-terminus of the protein, showed the presence of a supershifted complex (lane 8). Supershift analysis with the JunB antibody showed no shifted complexes (lane 9), while addition of the JunD antibody

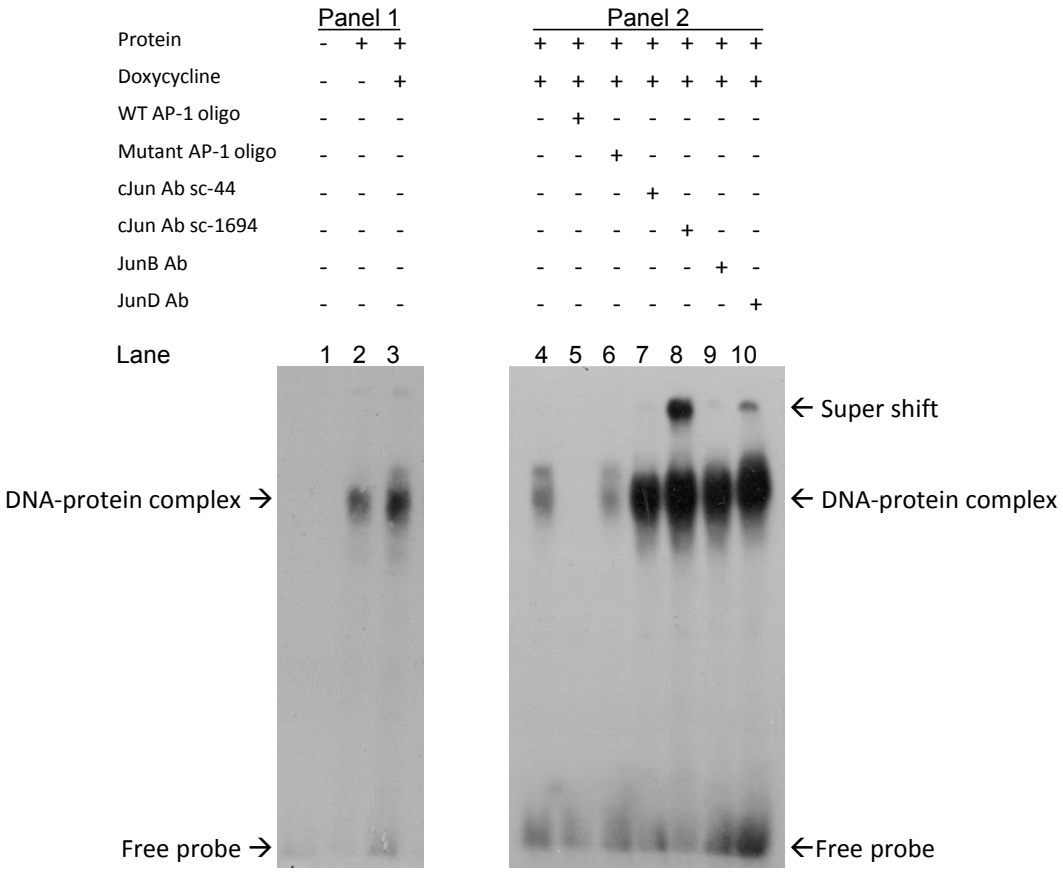


**Figure 2.9: AP-1 binds directly to the PAK3 promoter at (+52/+60) *in vitro*.** Electrophoretic Mobility Shift Assay (EMSA) utilizing a radioactively-labelled (+52/+60) PAK3 promoter region oligomer (free probe). **Panel 1** shows the presence of a DNA-protein complex when the labelled (+52/+60) PAK3 promoter oligomer was incubated with protein cell lysate from Rat1a-J4 cells (lane 2), and to a greater extent, when incubated with protein lysate from Rat1a-J4 cells treated with doxycycline for 48 hours (lane 3). **Panel 2** shows further analysis of the complex in the presence of 48 hour doxycycline induction of cJun/AP-1 in Rat1a-J4 cells. Lane 4, like lane 3, shows the presence of a DNA-protein complex when the labelled (+52/+60) PAK3 promoter oligomer was incubated with doxycycline-induced protein cell lysate from Rat1a-J4 cells. This DNA-protein complex could be competed for with unlabelled (+52/+60) wild-type (WT) oligomers (lane 5), but not with a mutated (+52/+60) sequence oligomer (lane 6). Lanes 7-10 show supershift analysis using two anti-cJun antibodies (against the DNA binding domain (lane 7), and against the N-teminus (lane 8)), as well as anti-JunB (lane 9) and anti-JunD (lane 10) antibodies.

was able to shift the DNA-protein complex (lane 10). These results show that AP-1 binds directly to the (+52/+60) AP-1 site in the PAK3 promoter *in vitro*, and cJun and JunD are involved in the active AP-1 complex transcriptionally regulating PAK3 expression in response to cJun/AP-1 over-expression.

*In vivo* binding of cJun to the (+52/+60) PAK3 promoter region was shown using Chromatin Immunoprecipitation (ChIP) Assays. Chromatin was harvested from Rat1a-J4 cells in the presence and absence of doxycycline treatment, cross-liked to any bound proteins and sonicated to generate DNA fragments of approximately 400 – 700 bp. Immunoprecipitation was done using a cJun antibody and protein G agarose beads. After a reversal of the cross-linking, the DNA was subjected to PCR using primers spanning the (+52/+60) AP-1 binding site.

In the presence of doxycycline-treated Rat1a-J4 cell extracts, the (+52/+60) region of the PAK3 promoter was successfully amplified from cJun pulled-down complexes (Fig. 2.10). A sample of the starting chromatin (Input DNA) served as a positive control for the presence of the (+52/+60) region, while chromatin immunoprecipitated without antibody served as a negative control. These results show that, *in vivo*, cJun/AP-1 binds directly to the (+52/+60) AP-1 binding site in the PAK3 promoter.

Together, these *in vitro* and *in vivo* assays confirm that cJun/AP-1 is a transcriptional activator of PAK3 expression through direct binding to the (+52/+60) region of the PAK3 promoter.

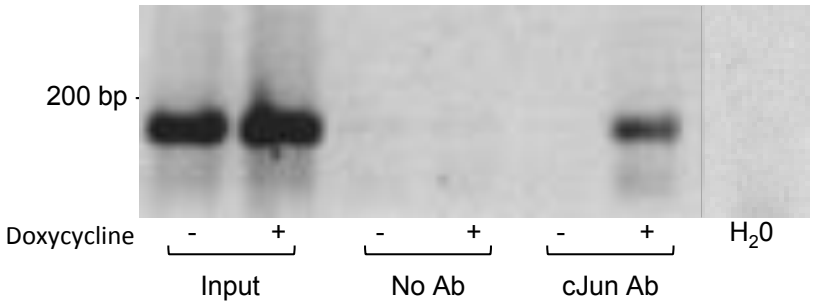


**Figure 2.10: AP-1 binds directly to the PAK3 promoter at (+52/+60) *in vivo*.** Chromatin Immunoprecipitation Assay (ChIP) showing cJun binding to the (+52/+60) PAK3 promoter region *in vivo*. Rat1a-J4 cells were treated with doxycycline for 48 hours, after which DNA and protein complexes were cross-linked and pulled down with an anti-cJun antibody (Ab). RT-PCR amplification of the starting chromatin samples, from both Rat1a-J4 cells with or without doxycycline, before pull-down (Input), as well as pulled-down chromatin in the presence of doxycycline, showed the presence of (+52/+60) PAK3 promoter region. The no antibody control and pull down in the absence of doxycycline showed no band after RT-PCR amplification with primers for the (+52/+60) PAK3 promoter region.

## 2.3. DISCUSSION

Despite the deregulation of AP-1 being known to play a pivotal role in transformation and tumourigenesis[142], very little is known about how it controls its diverse down-stream functions. AP-1 has been shown to target many genes, from intermediate filament markers like vimentin[143,144] and extracellular matrix proteins like fibronectin[55], to cell cycle regulators like p16 and p12[55]. Very few of AP-1's target genes have been investigated, or successfully implicated, in the AP-1-associated transformed phonotype. The studies that have identified involved genes were mostly performed in model systems using the inducible expression of AP-1 subunits, or homologues thereof, and gene expression analysis to identify differentially expressed genes in response to AP-1 over-expression.

DNA-microarray analysis in a mouse fibroblasts cell line, with inducible viral Jun (vJun) identified SSeCKS (Src-suppressed C kinase substrate) to be down-regulated in response to vJun over-expression[68]. Re-introduction of SSeCKS into vJun transformed fibroblasts reduced the foci and colony forming ability of the transformed cells[68], naming SSeCKS as a tumour suppressor[145].

The over-expression of vJun, in avian cell cultures, identified a homologue of the HB-EGF (Heparin-Binding Epidermal Growth Factor-like growth factor) to be over-expressed in transformed cells[67]. In chicken embryo fibroblasts, constitutive expression of HB-EGF resulted in the development of multi-layered foci and the ability to form colonies in anchorage-

independent growth conditions[67]. This noted HB-EGF as an effector in Jun-induced transformation.

The extra cellular matrix protein, SPARC, was also identified to be an AP-1 target gene involved in transformation. Secretion of the SPARC gene product has been shown to promote migration and invasion in breast and prostate cancer cell lines[146]. In rat fibroblasts with cJun over-expression, SPARC levels were decreased[147,148], while in an alternative cell model of a non-aggressive human breast cancer cell line, MCF7, the SPARC levels were seen to be increased[55]. This variation highlights the diverse role of AP-1 and its species- or cell-specific regulation.

The expression of a dominant negative Jun construct, TAM67, has been seen to block AP-1 transformation and tumourigenesis[149,150]. It was therefore hypothesized that TAM67 was capable of inhibiting genes critical to the transformed phenotype[69]. DNA microarray profiling recognized a number of genes, previously identified to be involved in tumour promotion and progression, that were inhibited by TAM67[69]. However, a unique target gene, Sulfiredoxin (Srx) was identified and shown to be a transcriptional target of AP-1 required for transformation[69]. Additionally, Srx was shown to contributed to a positive feedback regulation of cJun activity, suggesting it to be a significant AP-1 target gene that could be targeted for cancer prevention or treatment.

With the de-regulation of each of these target genes only leading to partial transformation, it is hypothesized that there is a group of AP-1 targets responsible for inducing the transformed

phenotype, as opposed to a single effector[13]. In order to identify additional AP-1 target genes necessary for cellular transformation, Kinoshita et al. (2003)[70], established a rat fibroblast cell line with inducible cJun expression and used microarray analysis to detect genes differentially expressed between control and cJun/AP-1 over-expressing cells. PAK3, a serine/threonine kinase signal transduction molecule, was identified as a potential AP-1 target gene. In this study, we have confirmed this data, showing PAK3 mRNA and protein expression to be up-regulated in response to cJun/AP-1 over-expression in both anchorage-dependent and -independent growth conditions. We also showed that PAK1 and PAK2 protein expression does not respond to cJun/AP-1 over-expression, suggesting that they are not AP-1 transcriptional targets. Since PAK3 is a previously undescribed AP-1 transcriptional target, this study is the first to shown the AP-1 regulation of Group I PAK protein.

We have identified various putative AP-1 binding sites in the (-2436/+149) rat PAK3 promoter region. This region was specifically responsive to cJun/AP-1 over-expression via an interaction with a site at position (+52/+60). In a follow-up study to the microarray that identified PAK3, the expression of PAK3 and 9 other candidate genes was further validated by Northern blotting[9]. Although it was hypothesized, these studies did not investigate whether these cJun-responsive genes were direct or indirect targets of AP-1 over-expression. Our data has now shown that PAK3 is in fact a direct transcriptional target of AP-1, with AP-1 binding directly, both *in vitro* and *in vivo*, to the (+52/+60) AP-1 binding site.

The AP-1 transcription factor binds one of two sites: the TPA responsive element (TRE)[32] or the cyclic AMP responsive element (CRE)[11]. The dimerization partners of AP-1 are important determinants in the preference AP-1 has for these binding sites. Jun:Jun and Jun:Fos dimers bind the TRE with a high affinity, while, depending on the flanking sequence, can also bind the CRE. Alternatively, Jun:ATF and ATF:ATF dimers favour the CRE[11,151]. The ATF:ATF dimers which are capable of binding the CRE are heterodimers of CREB and AFT1, however other AFT family members requires dimerization with a Jun subunit to bind the eight base-pair site of the CRE[11].

Through supershift analysis in the presence of cJun/AP-1 overexpression, we have shown that cJun and JunD are present in the AP-1 complex which binds the (+52/+60) site in the PAK3 promoter. Since this site is a classic CRE, which along with its flanking sequences, was conserved across species, it suggests there could be a number of AP-1 dimers which could result in the transitional activation of PAK3. Therefore, cJun and JunD, which we have shown to bind the (+52/+60) site, could be in the form of Jun:Jun, Jun:Fos as well as Jun:ATF dimers, where cJun and JunD act as dimerization partners to the ATF proteins. As cJun has been seen to form stable dimers with ATF2, ATF3 and ATF4, but not with ATF1 and CREB[11,152], further investigation into the AP-1 subfamiliy members involved in PAK3 activation, would be an interesting and novel finding. We have additionally shown that JunB is not involved in binding the PAK3 promoter region. Promoter activation by JunB has been reported to require a number of AP-1 binding sites, while cJun is able to activate promoters via a single site[35,36]. Since we have shown that AP-1 activation of PAK3 expression occurs via a single binding site, this supports our findings. There is also evidence showing cJun and JunD acting in a similar manner. cJun and

JunD have been shown to activate the protooncogene, c-myb, by binding to a single site in the promoter of the gene, while JunB did not bind the promoter nor activate transcription of this gene[153].

In conclusion, our data showed that PAK3 is an AP-1 target gene that is transcriptionally regulated through the direct binding of AP-1 to single site in the PAK3 promoter.

# CHAPTER 3

# IDENTIFICATION OF A FUNCTIONAL ROLE FOR PAK3 WITHIN TRANSFORMED AND CANCER CELLS

## 3.1. INTRODUCTION

cJun, the central component of the AP-1 transcription factor[12], was first discovered by its ability to transform cells[13]. A transformed cell refers to a mammalian cell that has acquired permanent disturbances in the control of cellular growth and/or locomotion[154] and gains a phenotypic change through the introduction of a new gene into the cell or a by a mutation in the genetic material of the cell[155]. The altered phenotypes seen in transformed cells include a loss of contact inhibition, a reduced requirement for growth factors (serum) and the ability to grow anchorage-independently[155]. As a result of these alterations there are changes in the appearance, adhesion and surface properties of transformed cells[156], where these changes are important for and mimic the process of tumour formation.

With AP-1's ability to transform cells, AP-1 was found to be highly involved in many cancers. Deregulation of AP-1 expression is linked to cancers from breast, endometrial, colon, lung cancers to ovarian and cervical[42–46,157]. Cervical cancer is the fourth leading cause of cancer death in females worldwide accounting for approximately 275 100 deaths in the year 2008[2]. Most of these deaths, 85% of them, occur in developing countries[158]. Cervical cancer is now the third most commonly diagnosed cancer worldwide, where South Africa has one of the highest incidence rates[2], largely due to the lack of effective screening programmes that allows for the

detection of the treatable precancerous lesions. Cervical cancer is a malignant growth arising from cells of the *cervix uteri*, the lower, narrow portion of the uterus (womb) where it joins to the vagina. The development of 99.7% of cervical cancer cases is caused by an infection with the Human Papilloma Virus (HPV)[159]. The HPV viral proteins, E6 and E7, transform the epithelial cells of the cervix by inactivating p53 and the Retinoblastoma protein, deregulating the cell cycle of these cells[160,161]. AP-1 has been seen to play a large role in the gene expression of the viral HPV proteins, controlling the oncogenic transformation and the tumourigenic phenotype of HPV-infected cervical cells[162]. It is thus not surprising that inhibition of AP-1 results in a reduction in HPV transcription and induction of apoptosis in cervical cancer cells[163].

In another gynaecological cancer, ovarian cancer, AP-1 also plays a distinctive role. Expression of all Jun family members were seen in both normal and cancerous ovarian tissue, with AP-1 playing a role in both the differentiation of normal ovarian surface epithelium and in the proliferation of ovarian cancer cells[43]. Ovarian cancer is the seventh leading cause of cancer related deaths worldwide, and was responsible for 140 200 deaths in 2008[2]. Since little is known of its tumour progression properties, it has one of the highest mortalities of gynaecological malignancies. The most common type of ovarian malignancy, accounting for 90% of all ovarian cancers, is surface epithelial tumours, or carcinomas[164]. These carcinomas are classified by the type of epithelia in the female reproductive tracks from which they arise, namely serous, mucinous, endometrioid, clear cell and Brenner tumours[164].

Serous ovarian cancers are by far the most common of all ovarian tumours, accounting for 50-60% of all ovarian carcinomas[165]. They are comprised of epithelium resembling that of the fallopian tube, or less often, from that of the surface of the ovary[166]. Although mutations in various genes, such as AKT2[167] and HER2/ERBB2[168], have been linked to high grade serous tumours, the primary genetic alterations associated with these carcinomas remain to be identified.

With AP-1 showing a strong link to the development of many cancers, including cervical and ovarian cancer, understanding the functional role of AP-1 and its targets genes in the transformed and oncogenic phenotype is of significance. In the previous chapter, we showed that PAK3 expression is elevated, at both the mRNA and protein level, in cJun/AP-1 transformed rat fibroblasts. As PAK3 expression is thought to be restricted to neural tissue[71], the up-regulation of PAK3 expression in transformed fibroblasts suggests that this expression may be of some functional importance to the transformed and oncogenic phenotype. However, the increased expression of PAK3 seen may also be a by-product of the elevated levels of AP-1 in transformed and cancerous cells, with no implications on the cancer cell biology.

In this chapter, we aim to determine the biological effect of inhibiting PAK3 expression in transformed rat and human fibroblasts, as well as investigate the level of PAK3 expression and function in human cancer systems, specifically the gynaecological cancers of cervical and serous ovarian cancer.

## 3.2. RESULTS

### 3.2.1. PAK3 inhibition does not play a role in the anchorage-independent growth advantage of transformed rat fibroblasts

To determine the role that PAK3 may play in the transformed phenotype, siRNA targeted against PAK3 was used to inhibit its expression in the presence of cJun/AP-1 induced expression in the rat fibroblast model system, and the biological effect assayed.

Rat1a-J4 cells were transiently transfected with 20 nM siRNA against PAK3, alongside 20 nM of control siRNA, and protein harvested after 24 and 48 hours. The control siRNA carries a scramble sequence with no known cellular mRNA target. The effect of the siRNA technology on the protein levels of PAK3 was assayed using western blot analysis. Our results showed a significant inhibition of PAK3 protein levels within 24 hours of transfection with PAK3 siRNA (Fig. 3.1). This inhibition of PAK3 was seen in the presence of cJun/AP-1 over-expression.

We next analysed the biological effects of PAK3 inhibition in cJun/AP-1 over-expressing cells. cJun/AP-1 transformation is associated with biological changes in proliferation, morphology and migration, and thus alterations in these characteristics were investigated when PAK3 was inhibited.

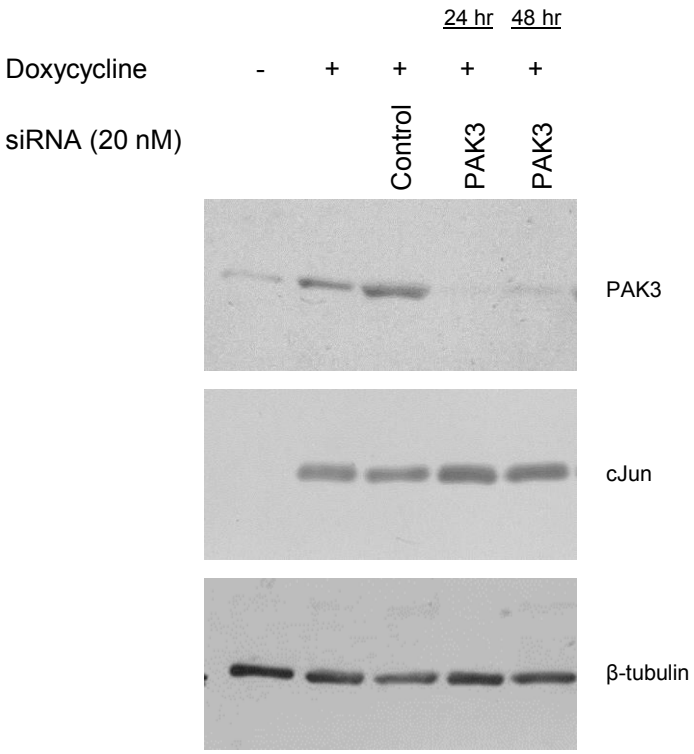


**Figure 3.1: Inhibition of PAK3 in cJun/AP-1 over-expressing cells.** Rat1a-J4 cells were transiently transfected with 20 nM PAK3 siRNA and treated with doxycycline to induced cJun/AP-1 over-expression. Protein was harvested after 24 and 48 hours and compared, via western blot analysis, to protein extracted from Rat1a-J4 cells in the absence and presence of doxycycline, with 20 nM control siRNA. This western blot analysis confirms transient PAK3 inhibition, even in the presence of doxycycline-induced cJun over-expression.

Cell proliferation assays in Rat1a-J4 cells, grown in the absence and presence of doxycycline induction of cJun expression, were performed for both anchorage-dependent (Fig. 3.2A) and anchorage-independent (Fig. 3.2B) growth conditions. The inhibition of PAK3 expression using PAK3 siRNA had no effect on cell proliferation. In the anchorage-independent growth conditions, cJun/AP-1 over-expressing Rat1a-J4 cells have a growth advantage characteristic of transformed cells. Inhibition of PAK3 had no effect on this growth advantage. These results suggest that PAK3 expression does not provide a proliferative advantage to transformed cells.

### 3.2.2. PAK3 plays a key role in the cell morphology and actin reorganization associated with AP-1-induced transformation

When cJun/AP-1 expression was induced in control rat fibroblasts, phase contrast microscopy showed distinctive changes in the normal cell morphology to elongated, smaller spindle-shaped, tightly-packed cells (Fig. 3.3A). Transfection with control siRNA had no effect on this altered morphology, however, when PAK3 was inhibited, the morphology was similar to that of control cells grown in the absence of doxycycline. Quantification of the changes in cell area showed that PAK3 inhibition in cJun/AP-1 over-expressing Rat1a-J4 cells resulted in a significant increase in cell area, comparable to the area of untransformed Rat1a-J4 cells (Fig. 3.3B). This illustrates that PAK3 plays a role in the altered morphology associated with cJun/AP-1 transformation, as inhibiting its expression caused a reversion in the cJun/AP-1 associated morphology.

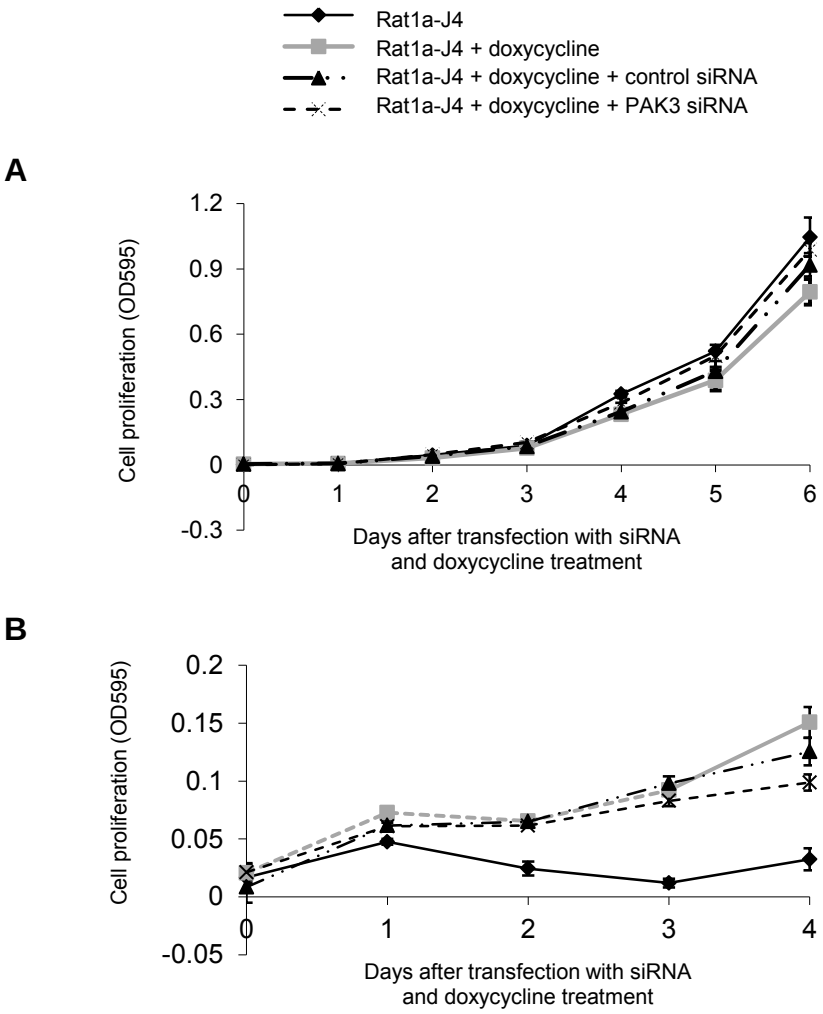


**Figure 3.2: Inhibition of PAK3 in cJun/AP-1 over-expressing cells has no effect on proliferation.** Cell proliferation assays (MTT) in doxycycline-induced cJun/AP-1 expressing Rat1a-J4 cells with control or PAK3 siRNA in anchorage-dependent **(A)** and anchorage-independent **(B)** growth conditions. The results shown are the mean ± S.E. of experiments performed in quadruplicate and repeated.

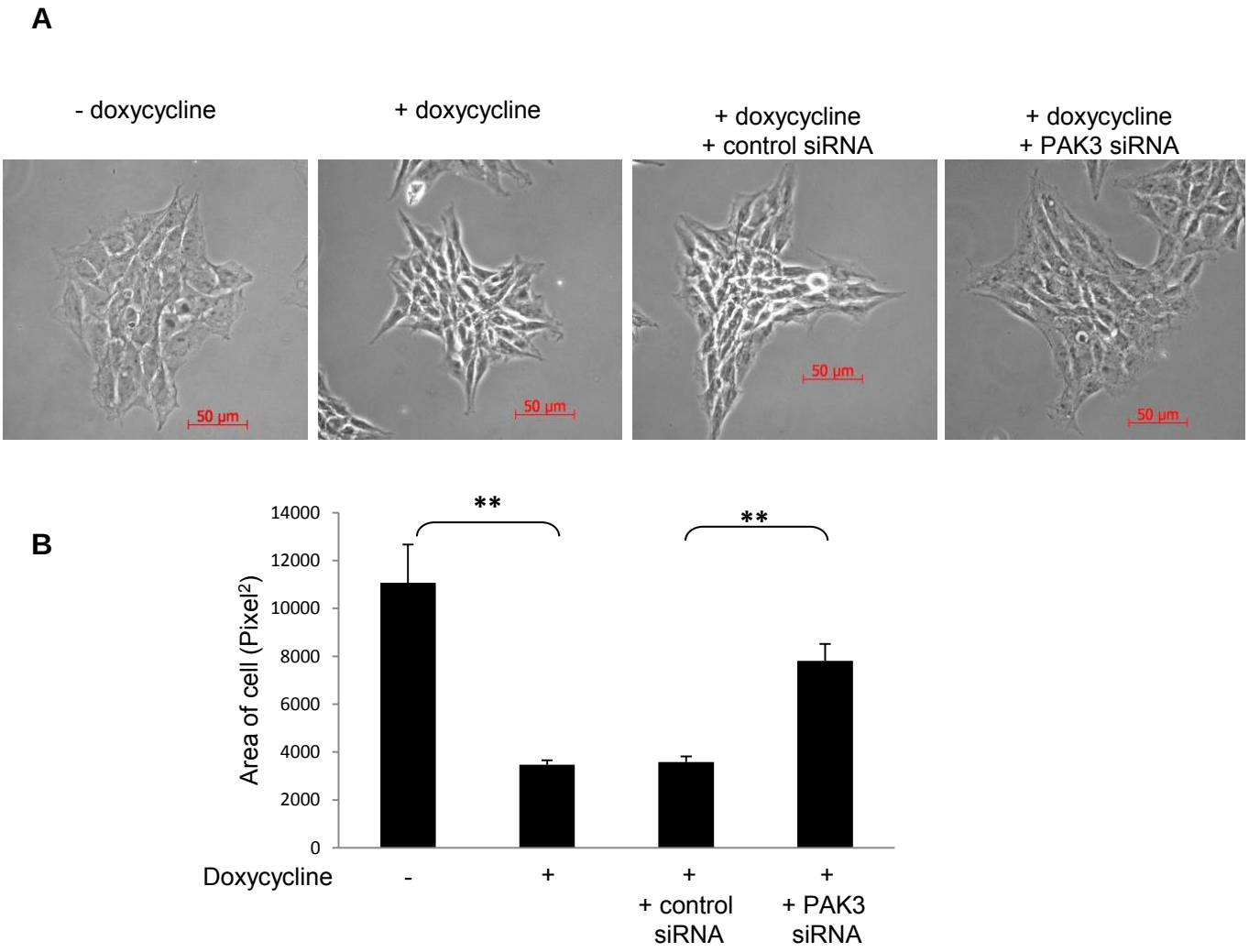


**Figure 3.3: Inhibition of PAK3 in cJun/AP-1 over-expressing cells results in a change in cell morphology.** **A:** Phase contrast microscopy of Rat1a-J4 cells grown in the absence and presence of doxycycline with the addition of either control or PAK3 siRNA. Pictures were taken 72 hours after transfection and treatment with doxycycline. **B:** The bar graph shows the mean area (pixel$^2$) $\pm$ S.E. of forty cells of each condition over four fields of view quantitated with AxioVision 4.7 software. (**p≤0.01).

Changes in cell morphology are often driven by cytoskeletal rearrangement. With PAK3 having been shown to be essential for the organization of F-actin at the leading edge of submarginal cells[169], we next investigated whether the morphological changes associated with PAK3 inhibition were linked to changes in actin redistribution. Actin staining assays were performed using phalloidin, which binds polymeric F-actin, to investigate actin reorganization in Rat1a-J4 cells when PAK3 was inhibited in the presence of cJun/AP-1 over-expression. This staining revealed an increased emergence of multiple actin-rich protrusions at the edges of the elongated, spindle-shaped cJun/AP-1 over-expressing Rat1a-J4 cells (Fig. 3.4A). Transfection with control siRNA had no effect on the cJun/AP-1-induced increase in actin rich cellular extensions. However, when PAK3 was inhibited there was not only a change in cell shape, but also a reduction in number of cellular protrusions. Quantification of these results showed that PAK3 inhibition in cJun/AP-1 expressing cells resulted in a significant increase in cell area and a significant decrease in the number of cytoplasmic extensions (Fig. 3.4B and C). This data confirms the changes in area seen in phase contrast microscopy. These results indicate that PAK3 plays a role in the actin reorganization associated with cJun/AP-1 transformation.

### 3.2.3. PAK3 plays a key role in the cellular migration associated with AP-1-induced transformation

Remodelling of the actin cytoskeleton is known to produce the inner motive force for the migration of cells[170]. Having observed that PAK3 inhibition has an effect on cell morphology and actin reorganization, Transwell motility assays were performed to investigate the potential role of PAK3 in cell migration.

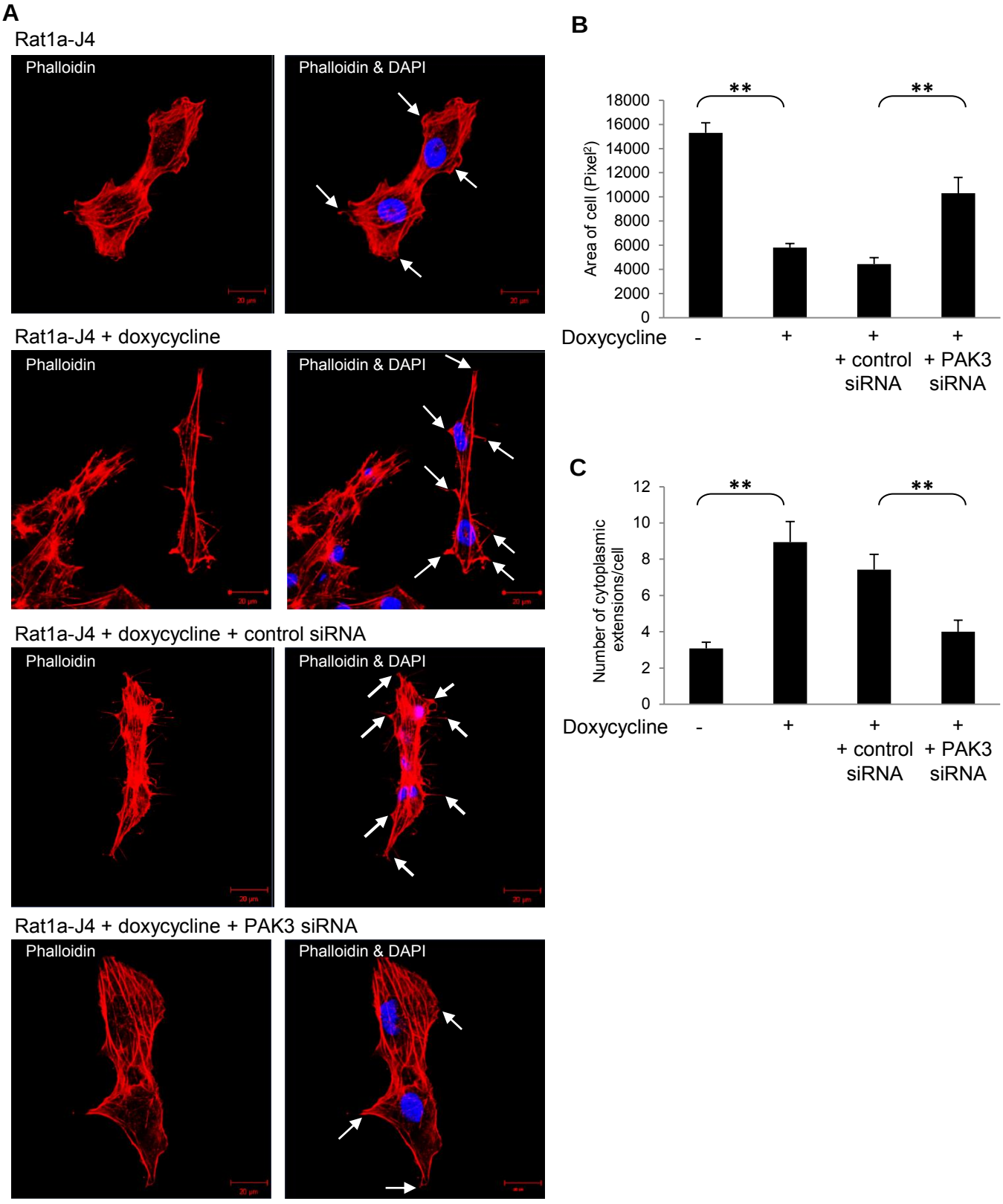


**Figure 3.4: PAK3 inhibition affects actin organization associated with cJun/AP-1 transformation. A:** Fluorescent staining of polymeric F-actin using phalloidin (red) in Rat1a-J4 cells grown in the absence and presence of doxycycline with control or PAK3 siRNA. DAPI stain was used to visualize the cell nuclei (blue). Arrows point to cytoplasmic extensions. **B & C:** Quantitation of the changes in cell area (pixel$^2$) (**B**) and number of cytoplasmic extensions per cell (**C**) from the captured fluorescent images. Cell area was calculated using AxioVision 4.7 Software and the cytoplasmic extensions were counted by eye. The results show the mean $\pm$ S.E. over six fields of view. (**p≤0.01)

For motility assays, Rat1a-J4 cells transfected with control or PAK3 siRNA were plated onto a porous membrane, in the presence or absence of doxycycline, above a well of media containing 15% serum as a chemoattractant. After 24 hours, the cells from the top of the porous membrane are removed with a cotton swab and the cells which migrated through the membrane to the other side are fixed, stained and quantitated. Motility assays showed that doxycycline-induction of cJun/AP-1 over-expression resulted in a significant increase in the migration of Rat1a-J4 cells (Fig. 3.5). Transfection with control siRNA had no effect on this increased migration, however, PAK3 inhibition reduced the migration of cJun/AP-1 over-expressing cells to a level comparable to cells grown in the absence of doxycycline. This result shows that PAK3 is required for the migration of cJun/AP-1 transformed rat fibroblasts.

### 3.2.4 PAK3 expression is elevated in transformed human fibroblasts

Having shown that elevated PAK3 expression, in response to doxycycline-induced cJun/AP-1 over-expression, associates with some of the phenotypes of AP-1 transformation in a rat fibroblast model system, we next investigated a role for PAK3 in transformed human fibroblasts. The normal embryonic lung fibroblast cell line, WI38, was used as a control and compared to its SV40-transformed counterpart cell line, SVWI38 cells.

PAK3 mRNA levels were found to be significantly increased in the transformed fibroblast cells, SVWI38, compared to the normal WI38 cells (Fig. 3.6A). Western blot analysis similarly showed increased PAK3 protein levels in the SVWI38 cells, compared to WI38 cells (Fig. 3.6B). The levels of cJun protein were also assayed and were found to be increased in the transformed SVWI38

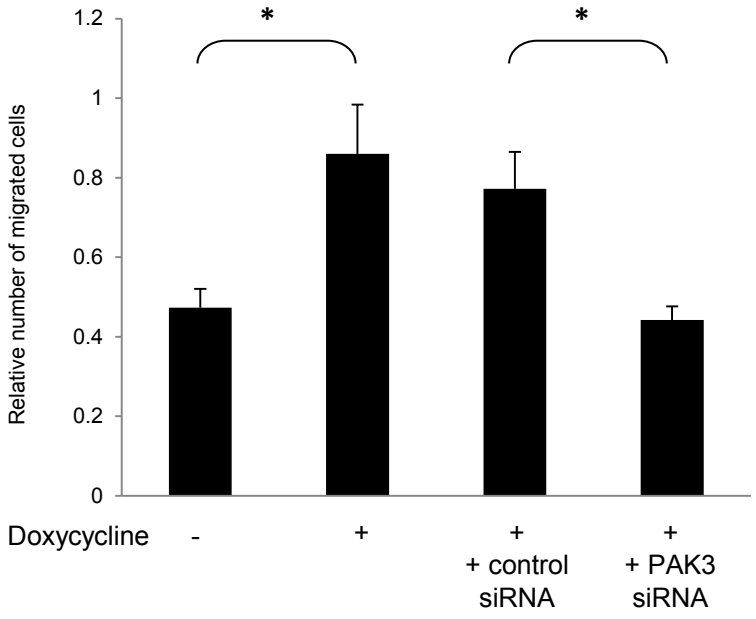


**Figure 3.5: PAK3 inhibition affects the migration associated with cJun/AP-1 transformation.** Migration assays, using Transwell chambers, were performed with doxycycline-induced cJun/AP-1 over-expressing Rat1a-J4 cells with either control or PAK3 siRNA. Cells transfected with siRNA and treated with doxycycline 24 hours prior were allowed to migrate for 24 hours after being platted onto the porous membrane of the Transwell Chamber. Cells that migrated through the membrane to the reverse side were then fixed and counted. The results show the mean $\pm$ S.E. of experiments performed in triplicate and repeated. (*p, 0.05)

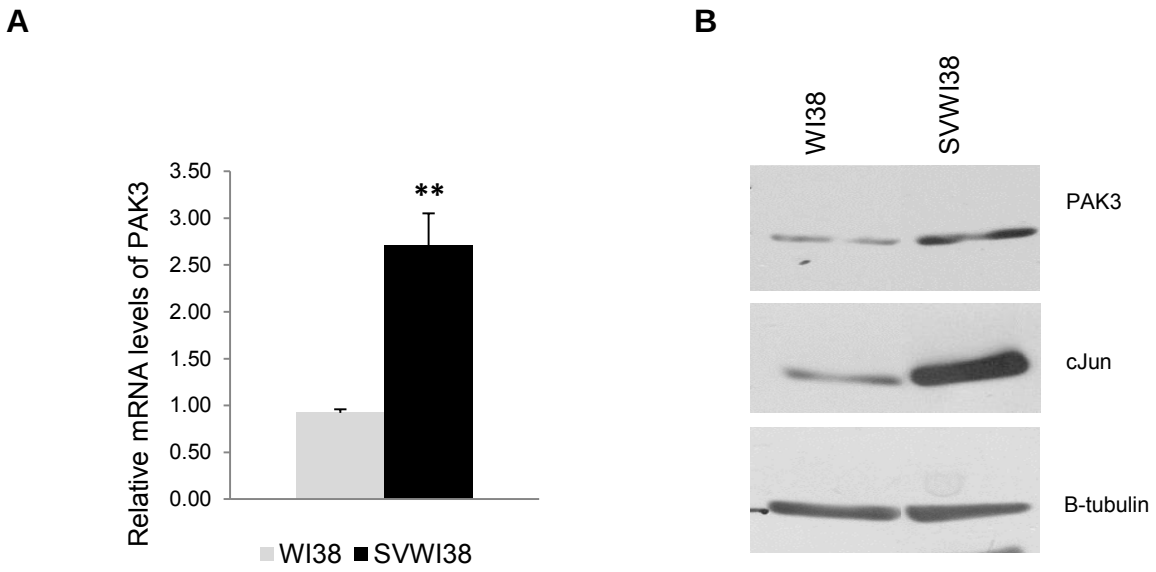


**Figure 3.6: PAK3 expression is elevated in transformed SVWI38 fibroblasts. A:** Real time RT-PCR for PAK3 mRNA expression levels in normal, WI38, and transformed lung fibroblasts, SVWI38. The expression level of PAK3 mRNA was normalized to the level of the house keeping gene, cyclophillin D, in each sample. The results show the mean $\pm$ S.E. of at three independent experiments. (**p≤0.01) **B:** Western blot analysis showing PAK3 and cJun expression levels from protein extracted from normal, WI38, and transformed lung fibroblasts, SVWI38.

cells. This result shows that elevated cJun/AP-1 and PAK3 protein levels associate with transformed SVWI38 cells and suggests a correlation between the increased expression of cJun and PAK3 protein in transformation.

### 3.2.5. Elevated PAK3 expression in human fibroblasts is transcriptionally dependent on cJun/AP-1 over-expression

In order to further investigate the correlation between cJun and PAK3 expression in human transformed fibroblasts, we assayed PAK3 promoter activity in WI38 and SVWI38 cells. The cells were transfected with the (-179/+149) PAK3 promoter reporter plasmid containing either the wildtype or the mutated AP-1 binding site at (+52/+60), and the luciferase activity measured. Transformed SVWI38 cells exhibited a significantly higher PAK3 promoter activity compared to the WI38 cells (Fig. 3.7A). Our results also showed that the AP-1 binding site at position (+52/+60) in the PAK3 promoter is required for the increased PAK3 promoter activity seen in SVWI38 cells. This results suggests that the increased PAK3 mRNA and protein level observed is due to the transcriptional activation of the PAK3 promoter by cJun/AP-1 via the (+52/+60) binding site.

To further address the necessity of the increased cJun expression on the elevation of PAK3 expression in SVWI38 cells, cJun protein levels were inhibited using siRNA technology. Western blot analysis showed that the reduction of cJun protein in response to cJun siRNA associates with a resultant decrease in PAK3 protein levels (Fig. 3.7B). This suggests that the increased levels of cJun appear necessary to maintain the increased level of PAK3 expression.

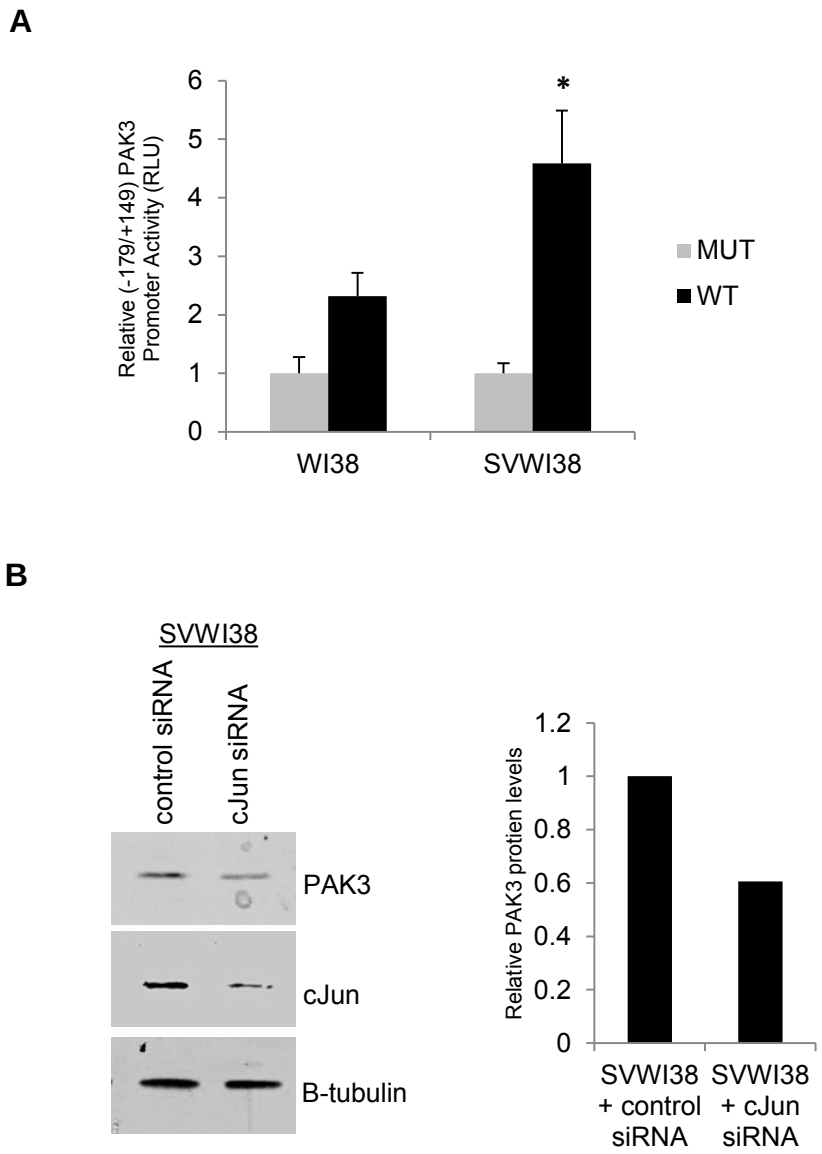


**Figure 3.7: Transcriptionally elevated PAK3 expression in SVWI38 fibroblasts is cJun dependent. A:** Luciferase reporter promoter assays in WI38 and SVWI38 cell lines transfected with the (-179/+149) PAK3 promoter reporter vector containing either a wildtype (WT) or mutated (MUT) cJun/AP-1 binding site at position (+52/+60). The promoter activity of the wildtype construct is expressed relative to the mutant construct activity for each cell type. Results show the mean $\pm$ S.E. of experiments performed in quadriplicate and repeated at three times. (*p≤0.05). **B:** Western blot analysis in SVWI38 cells with transient knock-down of cJun using siRNA, addressing the levels of PAK3 and cJun protein. The bar graph represents the quantitated levels of PAK3 protein relative to β-tubulin.

### 3.2.6. Inhibition of PAK3 expression in transformed human fibroblasts has no effect on proliferation, but plays a key role in morphology, actin reorganization and migration

siRNA technology was used to inhibit PAK3 protein levels. SVWI38 cells were transiently transfected with 20 nM control or PAK3 siRNA and protein was harvested 72 hours after transfection, when a change in the cellular morphology was observed. The effect of PAK3 siRNA was assayed using western blot analysis and showed a reduction of the PAK3 protein level seen in SVWI38 cells (Fig. 3.8A). Cell proliferation assays showed that PAK3 inhibition had no effect on the proliferative rate of SVWI38 cells (Fig. 3.8B), suggesting that PAK3 does not play a role in the proliferation of transformed human fibroblasts.

Phase contrast microscopy of WI38 and SVWI38 cells showed the characteristic phenotypes of the normal and transformed fibroblasts, however when PAK3 was inhibited in SVWI38 cells these small spindle-shaped cells became elongated, flattened and enlarged, resembling the morphology of the WI38 cells (Fig. 3.9A). Quantification of cell area from these results shows a significant increase in the cell area of SVWI38 cells with PAK3 inhibition, compared to normal SVWI38 cells (Fig. 3.9B). This result highlights a role for PAK3 in the altered morphology associated with transformation of human fibroblasts.

Analysis of actin reorganization using phalloidin to stain the F-actin in SVWI38 cells transfected with control or PAK3 siRNA supported the phase contrast microscopy data, showing a change in cell area (Fig. 3.10A). Control siRNA transfected SVWI38 cells appear to be much smaller,

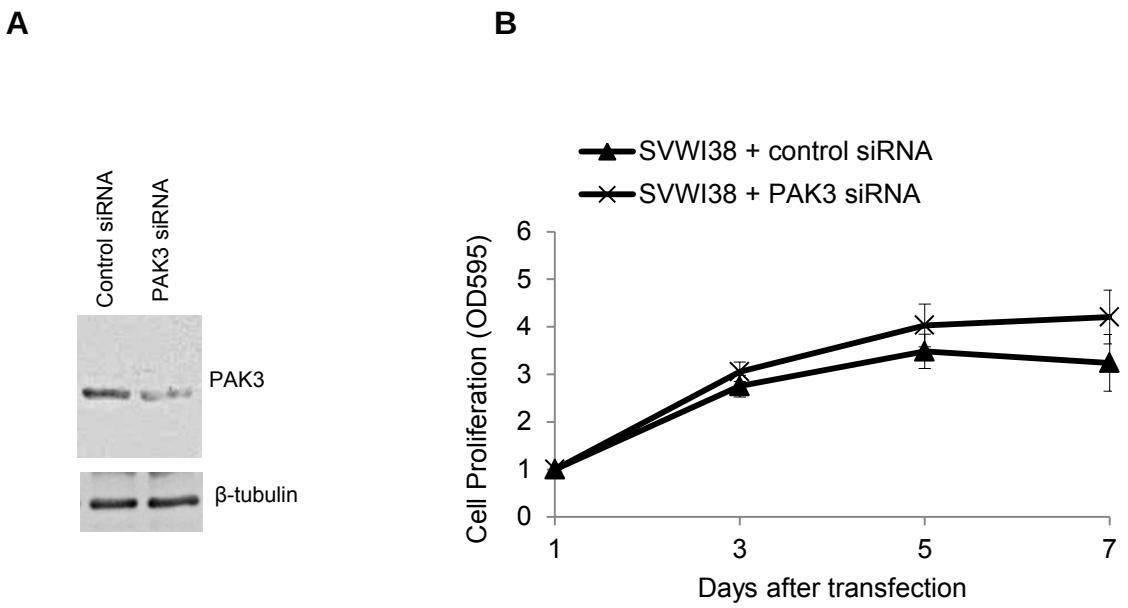


**Figure 3.8: Inhibition of PAK3 expression in transformed SVWI38 fibroblasts does not affect proliferation. A:** Western blot analysis showing PAK3 knock-down using siRNA in SVWI38 fibroblasts after 48 hours of transfection with control or PAK3 siRNA. **B:** Cell proliferation assays (MTT) for SVWI38 cells transfected with control or PAK3 siRNA. The results show the mean of the fold changes in absorbance $\pm$ S.E. of experiments performed in quadruplicate.

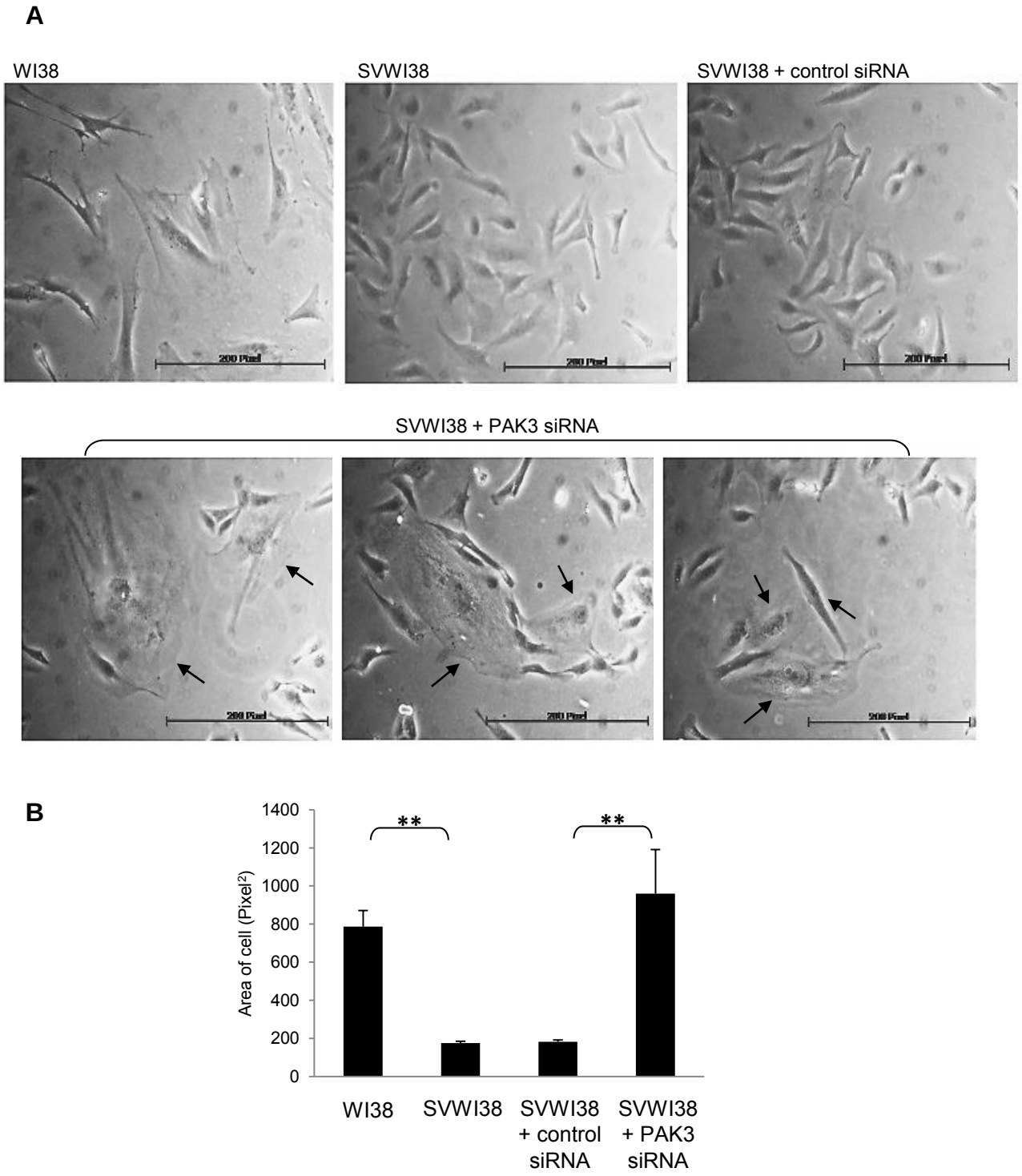


**Figure 3.9: PAK3 inhibition in transformed SVWI38 fibroblasts affects cell morphology. A:** Phase contrast microscopy showing the morphology of WI38 fibroblasts compared to SVWI38 fibroblasts transfected with control or PAK3 siRNA after 72 hours. **B:** The bar graph shows the mean area (pixel$^2$) $\pm$ S.E. of sixty cells of each condition over six fields of view quantitated with AxioVision 4.7 software. (**p≤0.01).

spindle-shaped cells with clear polarity, often with two major cytoplasmic protrusions stretching from the main cell body. However, PAK3 inhibited SVWI38 cells appear enlarged, rounded and often with loss in polarity. Quantitation of the changes in the cell area is shown in Fig. 3.10B. These results are supportive of those obtained from rat fibroblasts and indicate that PAK3 plays a role in the actin reorganization associated with cJun/AP-1 transformation.

With the effects of PAK3 inhibition seen on the morphology and actin reorganization of SVWI38 cells, Transwell motility assays were next used to assay the effect of PAK3 inhibition on the migration of SVWI38 cells. SVWI38 cells showed increased migration compared to their normal counter parts, WI38 (Fig. 3.11). Transfection with control siRNA has no effect on the migration of SVWI38 cells, while PAK3 inhibition significantly reduced the number of migratory cells to a level comparable to WI38 cells. This result shows that PAK3 is required for the migration of transformed human fibroblasts. Taken together, the above results show that PAK3 plays a distinctive role in the cellular transformation of fibroblasts cells from both human and murine origin. The biological phenotypes most affected by PAK3 include cell morphology, actin organisation and motility.

### 3.2.7. PAK3 protein levels are elevated in an array of cancer cell lines

Thus far, we have shown that PAK3 expression is elevated and plays a role in both transformed rat and human fibroblasts. As transformation mimics the process of cancer development[155], we hypothesized that PAK3 expression could play a significant role in the phenotype of human

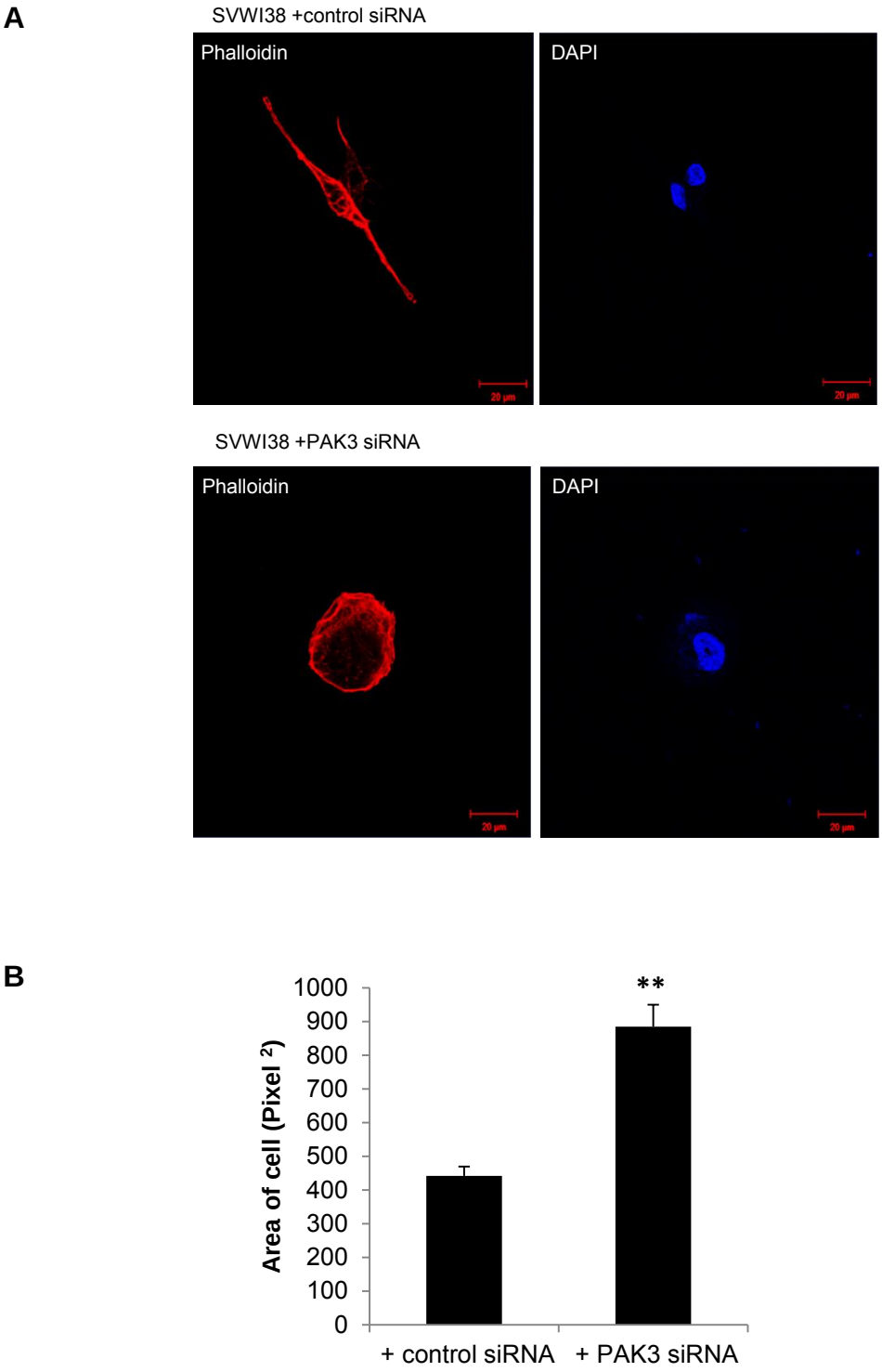


**Figure 3.10: PAK3 inhibition in transformed SVWI38 fibroblasts affects actin organization. A:** Fluorescent staining of polymeric F-actin using phalloidin (red) in SVWI38 cells transfected with either control or PAK3 siRNA showed cytoskeletal rearrangements. DAPI stain was used to visualize the cell nuclei (blue). **B:** Quantitation of the changes in cell area (pixel$^2$) from the captured fluorescent images. Cell area was calculated using AxioVision 4.7 Software. The results show the mean $\pm$ S.E. over six fields of view. (**p≤0.01)

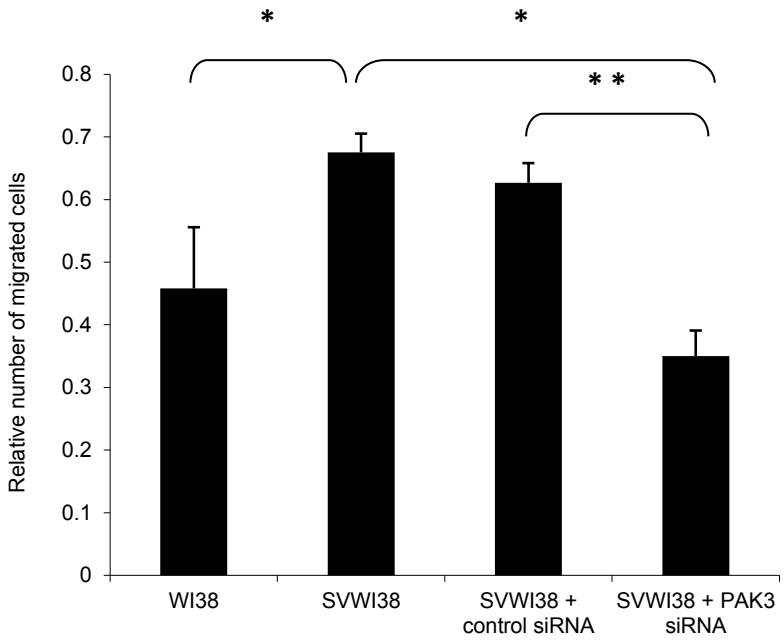


**Figure 3.11: PAK3 inhibition reduces the migration associated with transformed human fibroblasts.** Migration assays, using Transwell chambers, were performed with normal, WI38, and transformed human fibroblasts, SVWI38, transfected with control or PAK3 siRNA. Cells transfected with 20 nM siRNA 24 hours prior, were allowed to migrate for 24 hours after being platted onto the porous membrane of the Transwell Chamber. Cells that migrated through the membrane to the reverse side were then fixed and counted. The results show the mean $\pm$ S.E. of experiments performed in triplicate and repeated. (*$p \leq 0.01$, and **$p \leq 0.01$)

cancers. This in itself would be a novel finding as PAK3 expression was thought to be predominantly limited to the brain[71,130]. To investigate a potential role for PAK3 in human cancers, we determined PAK3 protein expression, using western blot analysis, in an array of available cancer and normal cell lines from different tissue origins.

Expression levels of PAK3 were determined in the cervical cancer cell lines: CaSki, HeLa, Me180, SiHa, Ms751 and C33A, and compared to a primary cervical epithelial cell line derived from normal cervical tissue, HCX (Fig. 3.12A). PAK3 protein levels appear to be elevated in all the cancer cell lines compared to the HCX normal primary cells, which showed negligible PAK3 protein expression. The cell line C33A, which is an HPV-negative cell line, also expresses PAK3 protein, suggesting that elevated PAK3 expression is not a consequence of HPV infection, but rather a more general feature of the oncogenic phenotype.

PAK3 protein levels were also investigated in an array of serous ovarian cancer cell lines: A224, A2780, OVCAR3, OVCAR433, SKOV3 and UC1107 (Fig. 3.12B). Unfortunately, no normal ovarian culture cell line was available with which to compare the cancer cell line expression levels, however, robust PAK3 protein expression was observed in the ovarian cancer cell lines assayed. GAPDH was used as a loading control, as β-tubulin has been seen to vary between ovarian cancer cell lines.

A panel of oesophageal cancer cell lines, along with a normal immortalized oesophgeal epithelial cell line, EPC2, was investigated for PAK3 protein expression (Fig. 3.12C). As with the

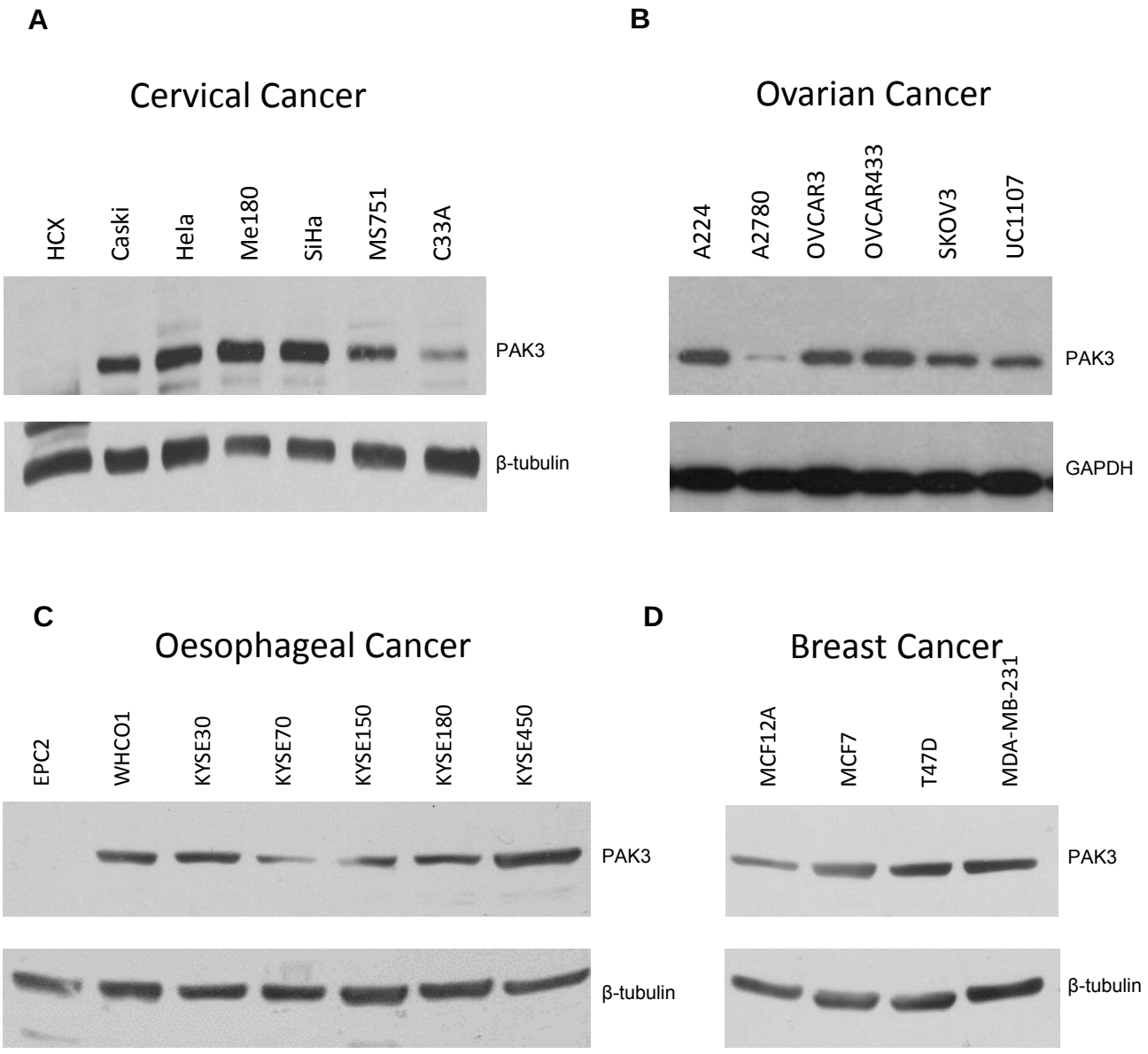


**Figure 3.12: PAK3 protein expression is elevated in an array of cancer cell lines.** Where possible, PAK3 protein levels in cancer cell lines were compared to normal or non-tumorigenic cell lines derived from the same tissue type as the cancer. **A:** Western blot analysis showing PAK3 protein expression levels in an array of cervical cancer cell lines, compared to PAK3 protein levels in a primary cervical epithelial cell line derived from normal cervical tissue, HCX. **B:** Western blot analysis showing PAK3 protein expression levels in an array of serous ovarian cancer cell lines. No normal ovarian tissue cell line was available in which to assay PAK3 expression. **C:** Western blot analysis showing PAK3 protein expression levels in an array of oesophageal cancer cell lines. In comparison, PAK3 levels were assayed in the EPC2 cells, a normal immortalized oesophageal epithelial cell line. **D:** Western blot analysis showing PAK3 protein expression levels in an array of breast cancer cell lines. Cancer cell line PAK3 expression levels were compared to expression levels in MCF12A cells, a non-tumorigenic mammary tissue cell line.

results obtained from the cervical cancer cell lines, PAK3 protein expression was increased in all the cancer cell lines, compared to the normal cells.

Lastly, the PAK3 expression levels in an array of breast cancer cell lines were compared to the levels of PAK3 protein in a non-tumourigenic mammary tissue cell line, MCF12A (Fig 3.12D). Results show that PAK3 levels were elevated in the breast cancer cell lines compared to their non-tumourigenic counterpart.

These results show that PAK3 protein levels were consistently elevated in all cancer cell lines assayed, showing that PAK3 expression is not only limited to neuronal tissue, but has a much wider range of expression than previously thought.

### 3.2.8. Inhibition of PAK3 in HeLa and A224 cells had no effect on proliferation, but significantly reduced the motility of the cancer cell lines

Having shown elevated levels of PAK3 protein in a number of cancer cell lines, we next determined whether this expression had any functional relevance in human cancers. Two cancer cell lines, representative of gynaecological cancers, were chosen in which to inhibit PAK3 and assay the effect: the cervical cancer cell line, HeLa, and the ovarian cancer cell line, A224. Since PAK3 expression has previously been seen in HeLa cells[171], this not only supports our data, but provides a positive control for PAK3 expression.

Plasmids containing short hair-pin RNA (shRNA) sequences targeting PAK3, alongside two control plasmids: the empty vector and a plasmid containing a scrambled sequence not

complementary to any other known cellular mRNA, were used. The generation of a stable shRNA transfectant was unsuccessful in HeLa cells and thus we utilized 20 nM of the shRNA in a transient transfection. Using real-time RT-PCR, our results showed sufficient inhibition of the PAK3 mRNA in the HeLa cells within 48 hours of transfection (Fig. 3.13A). PAK3 inhibition had no effect on the proliferation of HeLa cells (Fig. 3.13B), while a Transwell migration assays showed that there was significantly less migration in HeLa cells with PAK3 inhibition (Fig. 3.13C).

Stable transfection with the shRNA plasmids was more successful in the ovarian cancer cell line, A224. Western blot analysis using protein extracts from A224 cells stably transfected with the empty vector and the PAK3 shRNA containing vector showed that the PAK3 shRNA significantly reduced the PAK3 protein levels in A224 cells (Fig. 3.14A). β-tubulin could be used as a loading control in this experiment, as it was comparing protein extracts from the same parental cell line. As in HeLa cells, PAK3 inhibition had no effect on the proliferation of A224 cells (Fig. 3.14B), but significantly reduced migration in the cells (Fig. 3.14C).

These findings shows that the elevated PAK3 levels seen in the cancer cell lines HeLa and A224, does not play a role in proliferation, but plays a significant role in the motility of these cells. These data are in keeping with our data in transformed rat and human fibroblasts.

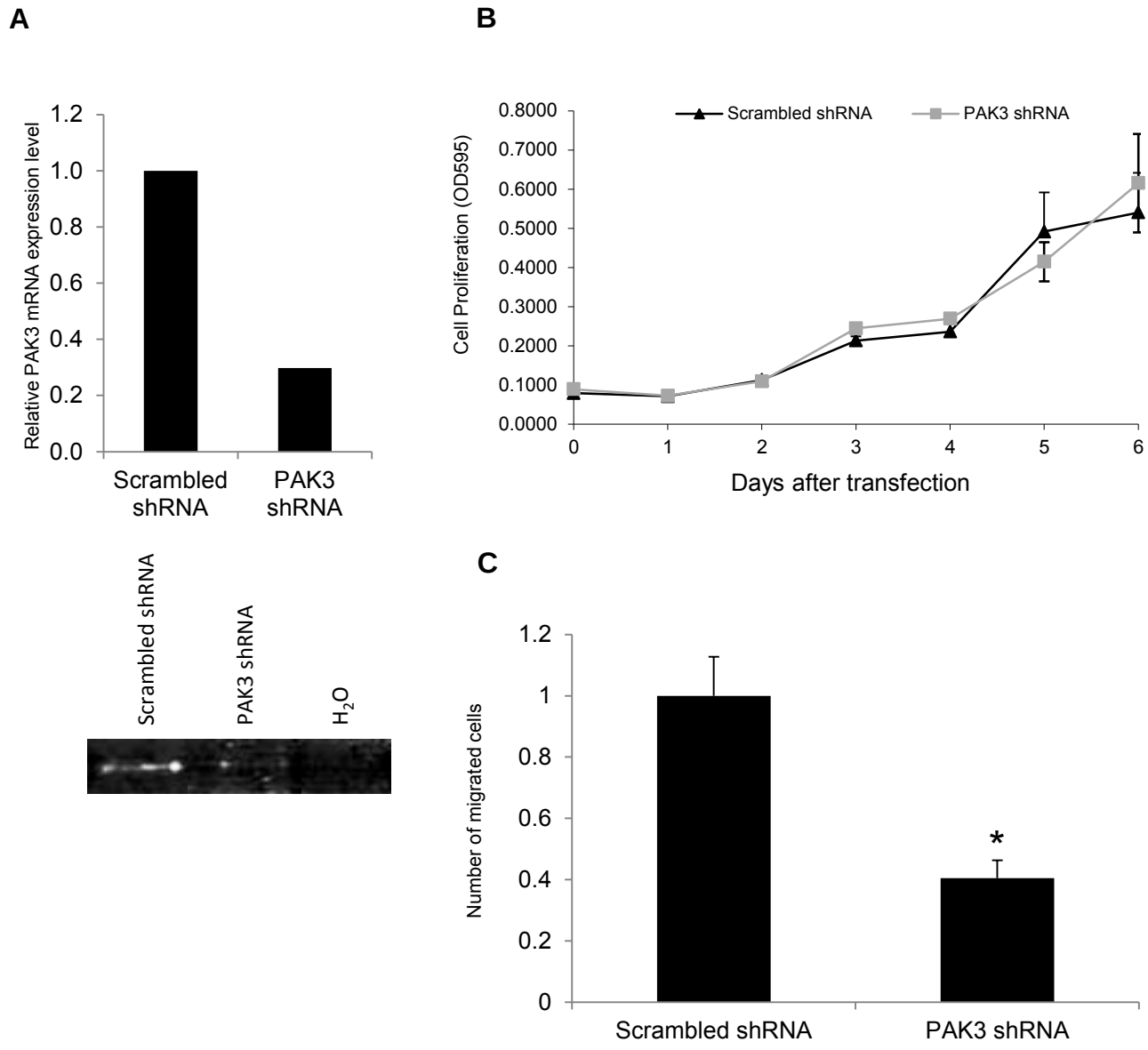


**Figure 3.13: PAK3 inhibition has no effect on proliferation, but plays a role in the migration of HeLa cells. A:** Real time RT-PCR analyses of PAK3 mRNA expression in HeLa cells transiently transfected with scrambled and PAK3 shRNA plasmids for 48 hours. The bar graph shows the relative expression levels on PAK3 relative to the house keeping gene, cyclophillin D. Amplification products were electrophoresed on a 1% agarose gel to show the knock-down of PAK3 mRNA. **B:** Cell proliferation assays (MTT) of HeLa cells transiently transfected with scrambled and PAK3 shRNA containing plasmids. The results shown are the mean $\pm$ S.E. of experiments performed in quadruplicate and repeated. **C:** Migration assays, using Transwell chambers, were performed with HeLa cells transfected with scrambled and PAK3 shRNA plasmids. Cells transfected with shRNA 24 hours prior, were allowed to migrate for 24 hours after being platted onto the porous membrane of the Transwell Chamber. Cells that migrated through the membrane to the reverse side were then fixed and counted. The results show the mean $\pm$ S.E. of experiments performed in triplicate and repeated. (*$p \leq 0.05$)

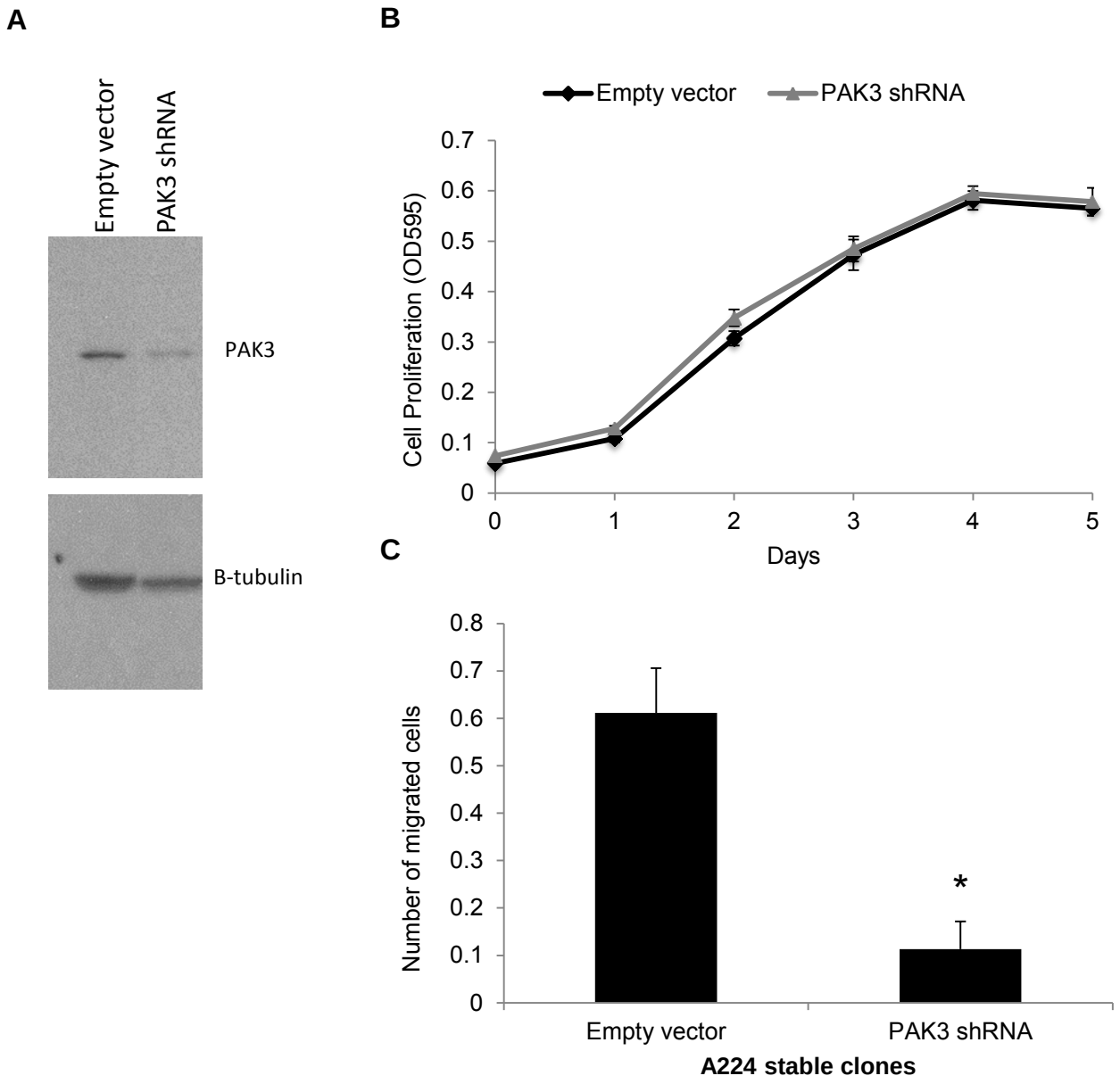


**Figure 3.14: PAK3 inhibition has no effect on proliferation, but plays a role in the motility of A224 cells. A:** Western blot analysis shows the PAK3 and cJun protein levels in A224 cells stably transfected with an empty vector and a PAK3 shRNA plasmid. **B:** Cell proliferation assays (MTT) of A224 cells stably transfected with the empty and PAK3 shRNA containing plasmids. The results shown are the mean $\pm$ S.E. of experiments performed in quadruplicate and repeated. **C:** Migration assays, using Transwell chambers, were performed with A224 staly expressing an empty vector and PAK3 shRNA plasmid. Cells were allowed to migrate for 3 hours after being platted onto the porous membrane of the Transwell Chamber. Cells that migrated through the membrane to the reverse side were then fixed and counted. The results show the mean $\pm$ S.E. of experiments performed in triplicate and repeated. (*$p \leq 0.05$)

### 3.2.9. PAK3 mRNA expression in cervical cancer patient biopsy material

Having shown that PAK3 was elevated in all assayed cancer lines and that PAK3 protein expression was necessary for the motility of HeLa and A224 cells, we next investigated PAK3 expression in biopsy material of cervical and ovarian cancer patients.

Quantitative real-time RT-PCR was used to measure PAK3 mRNA expression levels in RNA extracted from six normal and thirteen cervical cancer tissue specimens. PAK3 expression was normalised to the expression of the *β- glucuronidase* (gusB) and *cyclophilin D* genes, to account for the potential difference in RNA loading. These two normalizer genes were chosen as they displayed minimal variation in expression between the normal and cancer specimens and it has been suggested that more than one house-keeping gene be used when using human tissue biopsies[172]. A trend towards an increase in PAK3 gene expression in cervical cancer tissues, compared to normal tissues, was observed (Fig. 3.15). This trend was however, not significant, presumably due to the large variation in PAK3 expression in the cancer group. This variation could be due to the potential differences in the staging and subtypes associated with cervical cancer[173]. Single melt curves in the RT-PCR suggest that PAK3 mRNA is being expressed in adult cervical tissue even though PAK3 was thought to predominantly be expressed in the neurons and in embryogenesis[71,128]. This supports findings that PAK3 expression was detectable in a cervical cancer cell line[131].

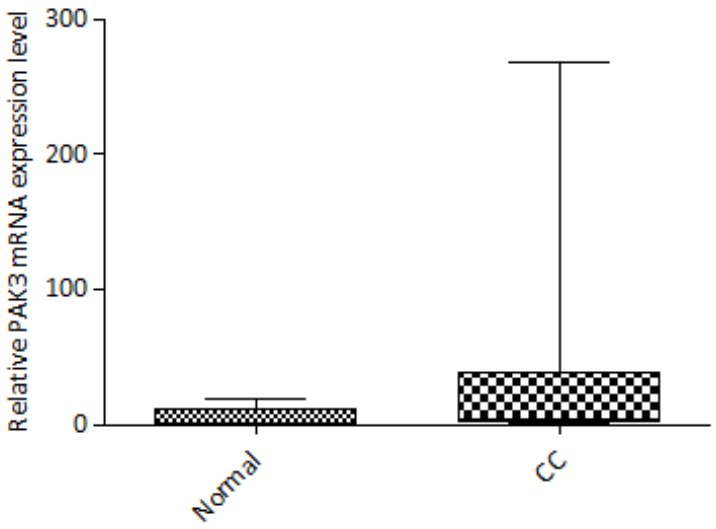


**Fig 3.15: PAK3 mRNA expression in normal and cervical cancer tissue.** Real-time RT-PCR analysis showing the mRNA expression of PAK3 in cervical cancer (CC) biopsies compared to normal cervical epithelium. Normal (n = 6), cancer (n = 15). The box and whisker graph was generated using Graphpad Prism. The results show the mean ± min/max.

### 3.2.10. High PAK3 expression tracks with poor survival in serous ovarian cancer

PAK3 expression was also investigated in another gynaecological cancer, serous ovarian cancer. Quantitative real-time RT-PCR was used to measure PAK3 mRNA expression levels in RNA extracted from five human ovarian surface epithelium (HOSE) samples, as the normal group, and forty-eight serous ovarian cancer tissue specimens. Our results showed that PAK3 mRNA levels were surprisingly significantly decreased in the ovarian cancer patient material compared to that in normal ovarian surface epithelial cells (Fig.3.16A). This result was perplexing as our western blot analysis, using a panel of ovarian cancer cell lines, showed high PAK3 expression in five out of six cell lines screened (Fig. 3.12B). A number of reasons could account for the low PAK3 expression observed in ovarian cancer patient specimens, including repression of gene transcription, increased protein degradation or changes in gene copy number.

With the chromosomal location of PAK3 being conserved to the X-chromosome and there being many molecular alterations that occur within the development of a cancer, often involving the X-chromosome, we looked at PAK3 DNA copy number changes among affinity-purified tumour cells from seventy-two primary high grade serous ovarian cancers. Using high-density single nucleotide polymorphism array, chromosomal instability index was measured by changes in DNA copy number. Overall, the frequency of the DNA copy number of the X-chromosome showed that the entire X-chromosome was more frequently deleted than it was amplified in serous ovarian cancer specimens. The region of the X-chromosome in which the PAK3 gene is located was found to be deleted in approximately 50% of the samples and only amplified in

14% of the samples (Fig. 3.16B). This result could account for the significant reduction seen in the PAK3 gene expression level in serous ovarian patient material.

Having shown that the PAK3 gene is often deleted in ovarian cancer patient material, we next performed a survival analysis to determine whether PAK3 expression levels are linked to the survival of patients with serous ovarian cancer. Survival analysis showed that patients with tumours with higher PAK3 levels had significantly poorer survival, compared to those with low PAK3 levels (Fig. 3.16B).

Taken together, these data suggest that PAK3 expression is linked to poor survival in ovarian cancer patients. PAK3, therefore, may serve as a potential prognostic marker for serous ovarian cancer.

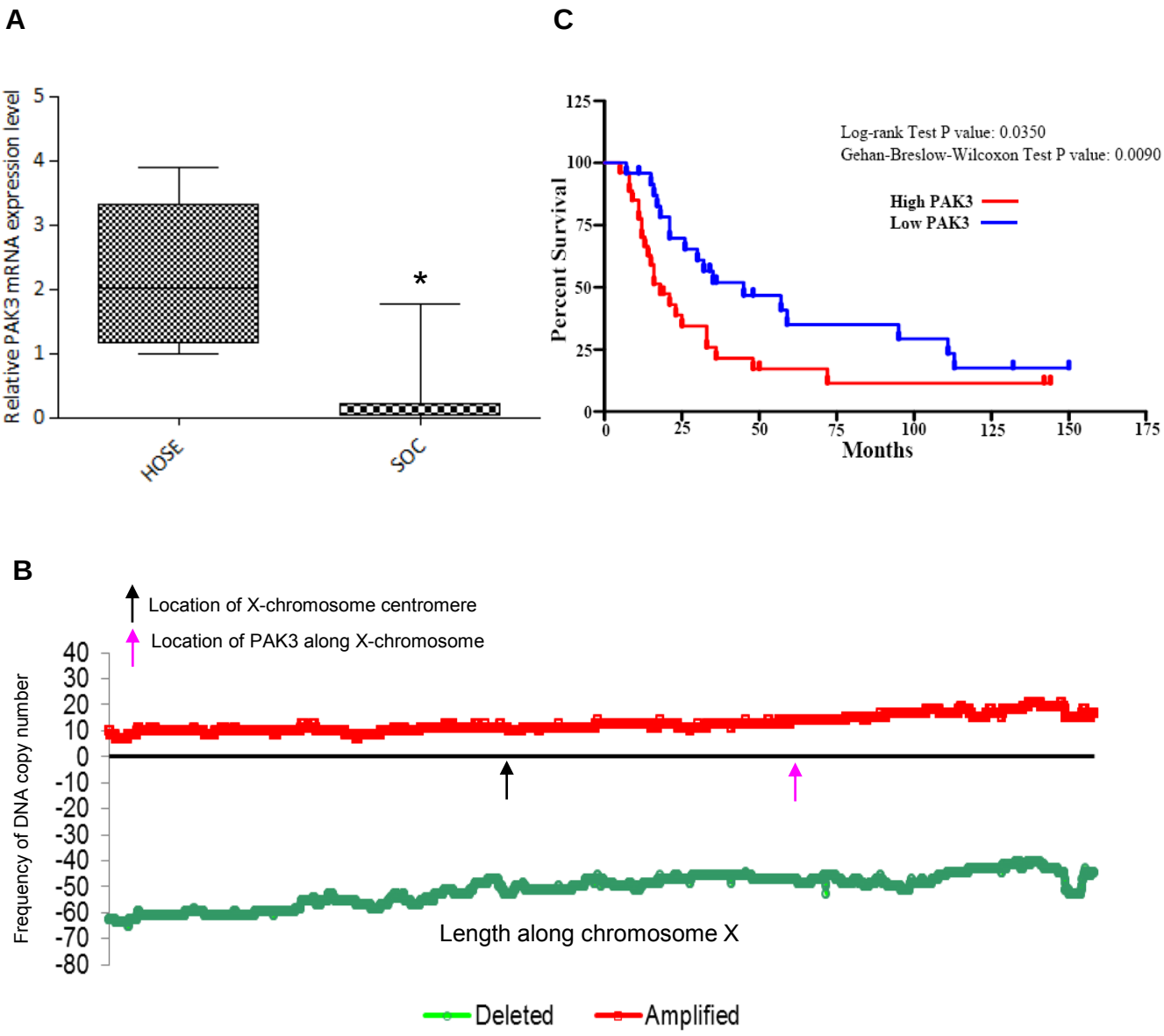


**Fig 3.16: PAK3 expression, copy number frequency and survival analysis in serous ovarian cancer tissue. A:** Real-time RT-PCR analysis showing the mRNA expression of PAK3 in serous ovarian cancer (SOC) biopsies compared to normal human ovarian surface epithelium (HOSE). HOSE (n = 5), cancer (n = 49). The box and whisker plot was generated using Graphpad Prism. The results show the mean ± min/max. (*p≤0.05) **B:** DNA copy number analysis of 72 primary high grade serous ovarian cancers showing the frequency of DNA copy numbers of the X-chromosome. The black arrow represents the location of the X-chromosome centromere, while the pink arrow marks the location of the PAK3 gene. **C:** Kaplan-Meier survival analysis for PAK3 expressing serous ovarian cancer patients with either low or high PAK3 expression. (p≤0.05)

### 3.3. DISCUSSION

In this chapter we show that PAK3 expression was elevated in human transformed fibroblasts, and that elevated PAK3 is dependent on elevated AP-1 expression. Furthermore, PAK3 expression was seen to be substantially elevated in cancer cell lines from different tissue origins, including cervix, ovary and breast. This result suggests that PAK3 has a far wider range of expression than previously thought. Negligible PAK3 expression was detected in the non-transformed and "normal" cell lines, compared to the cancer-derived cell lines, suggesting that PAK3 may play a functional role in oncogenesis.

Activated cJun is highly present in many carcinomas[174,175] and its activity is needed for Ras, a Rho GTPase, -mediated morphological transformation[176]. PAK3, through its dynamic interaction with Rho GTPases[79], is known to be involved in cell morphology[78]. Our study showed that inhibition of PAK3 in transformed rat and human fibroblasts repressed the transformed morphology and maintained a morphological phenotype more closely resembling control cells. Although the Rho family of GTPases are thought to activate the PAK proteins, it is specifically the Rho GTPases, CDC42 and Rac, that are the main activators of the PAKs[71]. CDC42 has been shown to activate filopodia formation, while the three isoforms of Rac (1, 2, 3) regulate actin polymerization at the periphery of the cell and control the development of membrane ruffles and lamellipodia[177–179]. Though the role of PAK proteins in the actin remodelling functions of CDC42 and Rac are undefined, PAK3, in particular, has been shown to be essential for the organization of F-actin at the leading edge of submarginal cells[169]. In this study we not only

show a role for PAK3 in the altered morphology of transformed cells, but also in the actin reorganization underlying these morphological changes. Our results suggest that PAK3 may be involved in the redistribution of F-actin to form actin-rich protrusions on the tips of the smaller, elongated transformed cells, as inhibition of PAK3 resulted in a significant decrease in the number of cellular protrusions and increased in the area of the cell. Our findings support work done by Amyere et al. (2000)[180] who showed that when Rat1a fibroblasts were transformed with an oncogene, such as K-Ras, one major or many cellular protrusions emerge from the cells and these protrusions show active actin-rich ruffling at the tips. Further analysis of these F-actin tips showed that these ruffles were in fact curled lamelliopodia. Other studies have shown that the activation of PAKs lead to the formation of lamelliopodia and motility of the cell[181–183], while PAK3 has specifically been implicated in actin filament regulation in proliferating cells[79,115]. Our results therefore implicate PAK3 specifically in the cytoskeletal reorganization of F-actin in transformed fibroblasts, and suggest that PAK3 induces the formation of lamelliopodia.

Remodelling of the actin in the cytoskeleton by regulatory proteins has been shown to play a distinctive role in cancer metastasis[184]. The Rho family of GTPases has also been shown to be vital regulators of cell movement in response to extracellular signals[185,186] and it is through the sequential activation of the family of GTPases that the coordinated control of cell motility comes about. For example, activation of CDC42 in Swiss 3T3 fibroblasts lead to the successive activation of Rac and Rho[177]. AP-1 activity is also known to mediate motility and invasion[54], where knock-down of AP-1 reduced directional migration[176]. Increased AP-1 activity was

observed to be directly linked to the highly aggressive phenotype of murine osteosarcoma cells, compared to their low metastatic counterpart[50]. PAK1 and PAK2, the other members of the Group 1 PAK family, are activated by cellular cues that stimulate migration[187] with PAK1 directly initiating the movement[79]. Since PAK3 inhibition affected actin reorganization in transformed cells, we hypothesized that PAK3 may play a role in the increased migration characteristic of cancer cells. Our results show that, in transformed rat and human fibroblasts, as well as in a cervical and ovarian cancer cell line, PAK3 inhibition significantly decreased cellular motility. This suggests that PAK3 plays a pivotal role in the migration of oncogenic and transformed cells.

While the inhibition of PAK3 had a significant effect on morphology and migration of transformed and cancer cell lines, this study showed it had no effect on the proliferative advantage seen in transformed fibroblasts, compared to their normal counterparts. Although this suggests PAK3 plays no role in proliferation, there is evidence to support that PAK1, PAK2 and PAK3 can play a compensatory role in proliferation[188]. Inhibition of the individual Group I PAK proteins was insufficient to inhibit increased proliferation, however simultaneous inhibition of all three PAK proteins successfully decreased the proliferation in neurofibromatosis type 2[188]. Thus, further inhibition of PAK1 and PAK2 proteins alongside PAK3 inhibition would give a clearer understanding of the role PAK3 plays in the proliferation of transformed and cancer cells.

While PAK1, 2 and 3 may play a compensatory role in proliferation, these proteins are not generally thought to have overlapping functions. However, PAK3 like PAK1, has been showed to

phosphorylate Raf1 and promote anchorage-independent growth: a characteristic of transformed cells[105,169]. Raf has in turn been shown to cause transformation of MDCK cells through the activation of Rac1[189]. The activity of PAK1 itself has been shown to be vital for Ras- and Rac-induced transformation[114,190]. PAK2 has also been linked to transformation, where AP-1 phosphorylation by PAK2 is required for the proliferation and anchorage-independent growth of transformed cells[191]. Due to problems associated with maintaining a stable PAK3 shRNA cancer cell line in this study, PAK1 and PAK2 levels were never measured in response to PAK3 inhibition. Due to the high level of homology in the Group I PAK proteins, future work identifying the interplay between PAK1, PAK2 and PAK3, in response to individual silencing, may identify other novel roles between these proteins, over and above their shared role in proliferation. Taken together, our data is the first to directly associate PAK3, a Group I PAK protein, with the transformed phenotype and demarcate a role for the elevated PAK3 expression in the morphology, actin reorganization and motility of the transformed cells.

With the PAK proteins playing a role in the transformed phonotype, it is not surprising that elevated expression and/or activity the other Group I PAKs have been seen in a number of tumours. Increased PAK1 expression has been shown in breast[192], colorectal[193] and ovarian cancers[194], while PAK2 activity is required for certain characteristics of prostate[195], ovarian[120] and breast cancer[119]. Having shown a role for PAK3 in the transformed phenotype, we assayed PAK3 expression in cervical and serous ovarian cancer patient material. We had previously shown that PAK3 expression was elevated in all cervical and the majority of serous ovarian cancer cell lines. While an increase in PAK3 expression was observed in cervical cancer

specimens, this however, was not significant. Serous ovarian cancer material surprisingly showed a significant decrease in PAK3 expression. Although the sample size may have had an impact on the data (our study had a relatively small sample size), the discrepancy between the PAK3 expression in cell lines versus tumour samples, suggests two possibilities: (i) that cancer cells with high PAK3 expression may have a selective advantage when grown in culture, or (ii) that PAK3 expression is lost in tumour samples.

Since PAK3 plays a role in the cytoskeleton and focal adhesions points[87], the elevated PAK3 expression seen in cancer cell lines could perhaps be an adaptive advantage gained by cultured cells to survive in a culture dish. The notion that PAK3 expression is related to cancer cells' ability to grow in culture is supported by data that found PAK4 to be over-expressed in a broad range of cell lines derived from a number of cancers such as lung, ovary, CNS, leukaemia, colon, renal, melanoma and breast[121]. The PAK4 gene and chromosomal locus have been reported to be amplified in a subset of human tumours[121]. PAK1 had a 59% DNA copy number gain in 30% of ovarian cancer material and resulted in the elevated expression of PAK1 in 85% of ovarian tumours[194]. We showed in ovarian patient material that the X-chromosome, where PAK3 is located, was deleted with a high frequency. This would explain the significant reduction in PAK3 protein levels in ovarian cancer patient material. Data by Hoque *et al.* (2008)[134] showed that there was a high frequency of cancer specific methylation of PAK3 and the X-chromosome in cancers of the oesophagus, lung, cervix, bladder and head and neck cancers, while PAK3 was only occasionally methylated in normal tissues. Together with our data, it appears that PAK3

expression, due to its location on the X-chromosome, could be silenced or decreased in tumour samples.

Interestingly, our data showed that high PAK3 expression in ovarian tumours was directly linked to significantly poorer patient survival. As PAK3 plays a role in the motility of cells, and with metastasis being the most life-threatening of cellular functions of clinically significant tumours[4], it would be interesting to investigate the level of motility in high and low PAK3 expression tumour samples. Elevated expression of both PAK1 and PAK2 are also associated with poor overall survival of ovarian cancer patients[120] and, like PAK1, perhaps PAK3 could serve a prognostic marker for cancer progression.

Along with the functional data suggesting a role for PAK3 in tumourigenesis, the finding that the PAK3-containing chromosomal region is often deleted on ovarian cancer, is indeed interesting. Since copy number analysis was only performed in ovarian cancer tissues, it certainly aids in the explanation of heterogeneity in PAK3 expression in the ovarian cancer tumours, however this data cannot be applied to other cancer types. Therefore, the functional relevance of such silencing cannot yet determined. However, in ovarian cancer tumours, despite PAK3 silencing in many of the patients, PAK3 still plays a significant role in those patients that do have PAK3 expression, potentially due to the functional role descibed in this chapter.

In summary, our results showed elevated PAK3 expression in all assayed transformed and cancer cell lines, and that this increased expression is vital to maintain the morphological and

migratory phenotype associated with transformation. Although the expression of high PAK3 did not correlate well with cervical and ovarian patient material, elevated PAK3 expression was linked to poor survival in ovarian cancer patients. Taken together, these data suggest a novel regulation of PAK3 in gynaecological cancers.

# CHAPTER 4

# INVESTIGATION OF A NOVEL FEED-BACK LOOP BETWEEN PAK3 AND AP-1

## 4.1. INTRODUCTION

PAK3 is thought to play a predominant role in the embryogenesis of the spine and brain, with PAK3 expression being mainly limited to neuronal tissue[71,128,130]. In the previous chapter, we showed that there were increased levels of PAK3 in an array of cancer cell lines from various tissues, compared to their normal counterparts. The expression of PAK3 in cancer cell lines, regardless of the tissue type, suggests a novel mechanism acquired by cancer cells to express PAK3.

In the well-known review, *The Hallmarks of Cancer*, Hanahan and Weinberg (2000)[4] summarize the six essential cellular alterations necessary for malignant growth, namely: self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of apoptosis, limitless replication potential, sustained angiogenesis and lastly, the ability to invade and metastasize[4]. Some years later, they published a follow-up review adding two more hallmarks to the list: reprogramming of the cellular energy metabolism and the evasion of immune destruction[5]. In both reviews, despite being separated by a decade of research, Hanahan and Weinberg state that the hallmark in which cancer cells are able to generate their own signals, rendering themselves self-sufficient, was arguably the most fundamental of all the hallmarks.

The ability of cancer cells to deregulate signals that are tightly controlled by normal cells enables cancer cells to override normal behaviour. Normal cells require a mitogenic growth signal, transmitted through transmembrane receptors and internal signal cascade pathways, to allow them to enter the cell growth and division cycle. By deregulating, or self-stimulating these pathways, cancer cells greatly reduce their dependence on external signals. The ability of cancer cells to sustain their proliferative signalling, and signalling required for processes such as motility and invasion, may be achieved in various ways. One such way may be as a result of constitutive activation of components of the signalling pathways, downstream from the receptors, removing the need to stimulate these receptors to activate the pathway. Deregulation of AP-1 activation has been observed in many cancers[42–47], suggesting that it could be the source of one such self-sufficient signal.

The transcription factor, AP-1, is activated when its subfamily members are phosphorylated and dimerise to form an active complex. This phosphorylation mainly occurs at the serine 63 and 73 residues in the Jun subunit and is controlled by the cJun N-terminal Kinase (JNK)[13,23]. ERK 1/2 have also been seen to perform this serine 63/73 phosphorylation[24]. Alternatively, other kinases, such as PAK2, have been shown to activate AP-1 through phosphorylation of alternative Jun residues or subunits[191]. However, thus far, JNK is considered to be the main activator of AP-1. The JNK proteins are activated through the MAPK pathway by a variety of stresses, such as UV, irradiation, oxidative stress, as well as pro-inflammatory cytokines[126]. Activated JNKs are phosphorylated and in turn phosphorylate a number of targets, from transcription factors like AP-1, to cytoskeletal molecules.

We have shown, in chapter 2, that AP-1 directly regulates PAK3. However literature has shown the PAK proteins, upstream of the of the MAPK/JNK pathway, activate the signal cascade responsible for activating AP-1[12,19,196]. Although the mere positioning of PAK proteins up-stream of AP-1 does not imply that PAK proteins are capable of activating AP-1, inhibition of PAK1 activity has been seen to block JNK/AP-1 activation[197], whereas AP-1 phosphorylation by PAK2 increased AP-1 activity[191]. In this chapter we questioned what effect PAK3 activation would have on AP-1. Unlike PAK3, PAK1 and PAK2 were not identified as AP-1 target genes, since their expression was not elevated in response to cJun/AP-1 over-expression (chapter 2). Should PAK3, as an AP-1 target gene, effect the activation AP-1, there may be the potential of a novel signalling feedback loop between PAK3 and AP-1. In this case, this feedback loop may play a role in contributing to the sustained signalling and phenotype of cancer cells, and account for the novel expression of PAK3 in cancer cell lines.

Here we investigated the potential of an interaction between PAK3 and AP-1. Having already characterized the regulation of AP-1 on PAK3, we next addressed a potential role of PAK3 activation on AP-1. To investigate this, we expressed constitutively-activated PAK3 and assayed the effects of activated PAK3 on the phosphorylation and activation of cJun, as well as intermediate kinases.

## 4.2. RESULTS

### 4.2.1. Expression of constitutively-activated PAK3

In order to investigate the potential of an interaction between PAK3 and AP-1, a constitutively-activated mouse PAK construct was used. The plasmid containing the activated PAK3 construct was kindly received from Dr Alan Horwitz (University of Virginia, USA)[198]. The constitutively-activated PAK3 contains a mutation in the threonine at position 421 to a glutamic acid, mimicking activation of the kinase by autophosphorylation at this site. Although activation of PAKs is characterized by the autophosphorylation on several sites, phosphorylation of the 421 threonine residue directly correlates with PAK activation[104].

The plasmid containing constitutively-activated PAK3 was sequenced confirmed, and the data aligned, using Multalin, with the wildtype (WT) mouse PAK3 mRNA sequence from the NCBI database. This alignment confirmed the presence of the activating mutation in the constitutively-activated (CA) PAK3 plasmid, converting the wildtype threonine (T) amino acid to a glutamic acid (E) at position 1263 in the mRNA (Fig. 4.1).

Having confirmed the presence of the activating mutation, western blot analysis was used to confirm expression of CA PAK3 in both SVWI38 and HeLa cells, representing transformed and cancer cell lines respectively. The CA PAK3 cDNA was cloned into the pCMV-myc vector, allowing us to differentiate endogenous PAK3 from the ectopically expressed CA PAK3 as the addition of a myc tag to the CA PAK3 protein resulted in an approximate 1.2 kDa increase in the

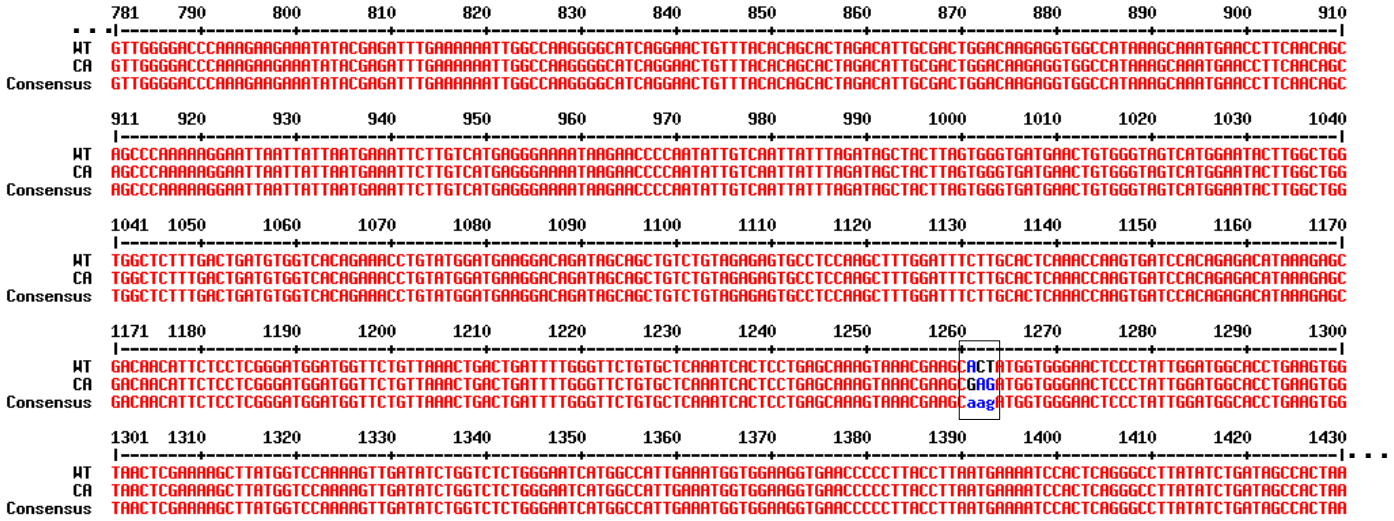


**Fig 4.1: Sequence alignment showing the incorporation of a constitutively activating mutation in PAK3.** A plasmid containing mouse PAK3 mRNA having undergone site-directed mutagenesis to generate constitutively active PAK3 was received cloned into the pCMV-myc vector. Multalin was used to align the wildtype (WT) PAK3 sequence along with the sequence data confirming the incorporation of the constitutively active (CA) mutation (T421E, base pair location 1263) in the PAK3 mRNA.

protein size, allowing the visualisation of CA PAK3 compared to the lower band of endogenous wildtype PAK3. Western blot analysis of protein extracted from SVWI38 and HeLa cells transfected with the empty vector, pCMV, or pCMV-myc-CA-PAK3, confirmed expression of the activated protein (Fig. 4.2A and B).

### 4.2.2. Expression of constitutively-activated PAK3 has no effect on the proliferation of SWI38 and HeLa cells

A proliferation assay was performed after transfection of SVWI38 and HeLa cells with the empty vector or CA PAK3, and results showed that CA PAK3 had no effect on cell proliferation (Fig. 4.3A and B). This is in keeping with our previous data, where the inhibition of PAK3 did not affect proliferation and confirms that PAK3 does not have a role in the proliferation of transformed or cancer cell lines.

### 4.2.3. Constitutively-activated PAK3 expression results in actin re-organization in SVWI38 and HeLa cells

We next evaluated the effect of CA PAK3 on the actin reorganization of SVWI38 and HeLa cells, as we had previously shown that PAK3 inhibition resulted in actin redistribution in transformed rat and human fibroblasts. Phalloidin was used to stain the polymeric F-actin in SVWI38 and HeLa cells transfected with the empty vector, pCMV or pCMV-myc-CA-PAK3. Results show that SVWI38 (Fig. 4.4A) and HeLa (Fig. 4.4B) cells transfected with the empty vector, pCMV, have an even distribution of actin across/surrounding the cell. However, in CA PAK3 expressing cells, the polymeric actin appears to be recruited to opposite ends/tips of the cells. This result

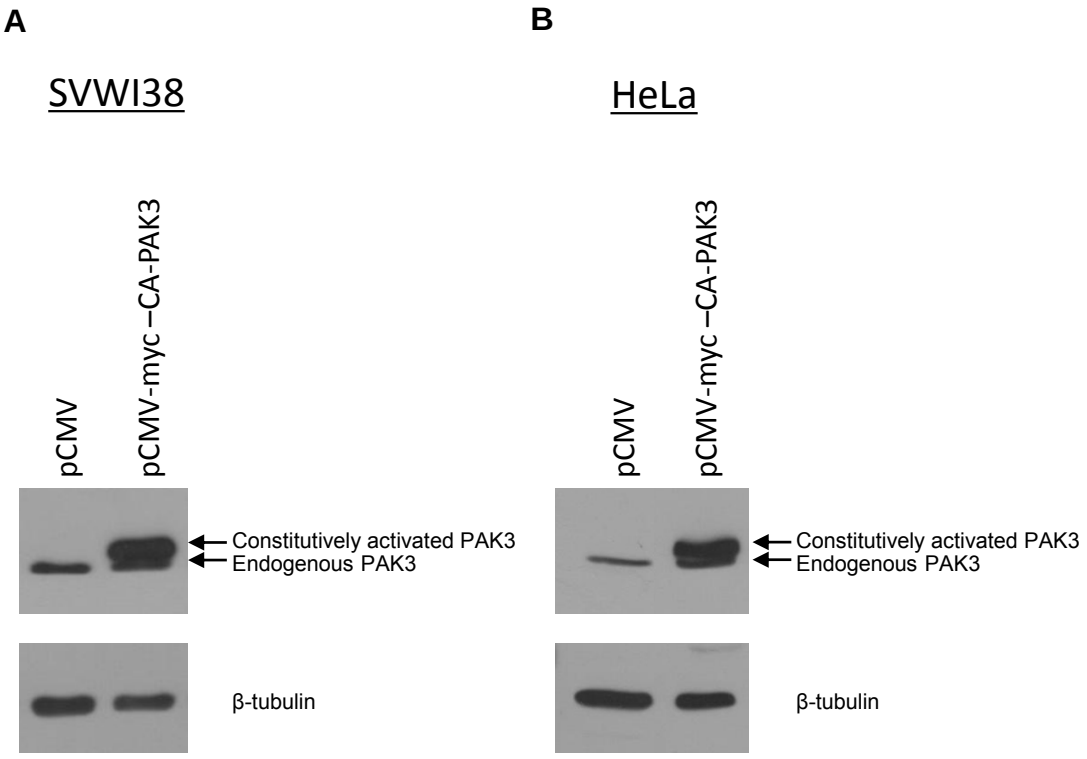


**Fig 4.2: Expression of CA PAK3 mutant in SVWI38 and HeLa cells.** Western blot analysis showing the protein expression levels of endogenous PAK3 and myc-tagged constitutively active (CA) mouse PAK3 protein in transformed human fibroblasts, SVWI38 (**A**), and cervical cancer cells, HeLa (**B**). Cells were transfected with 300 ng/ml of the empty vector, pCMV, or the CA PAK3 plasmid, pCMV-myc-CA-PAK3, and protein was harvested 48 hours later for western blot analysis.

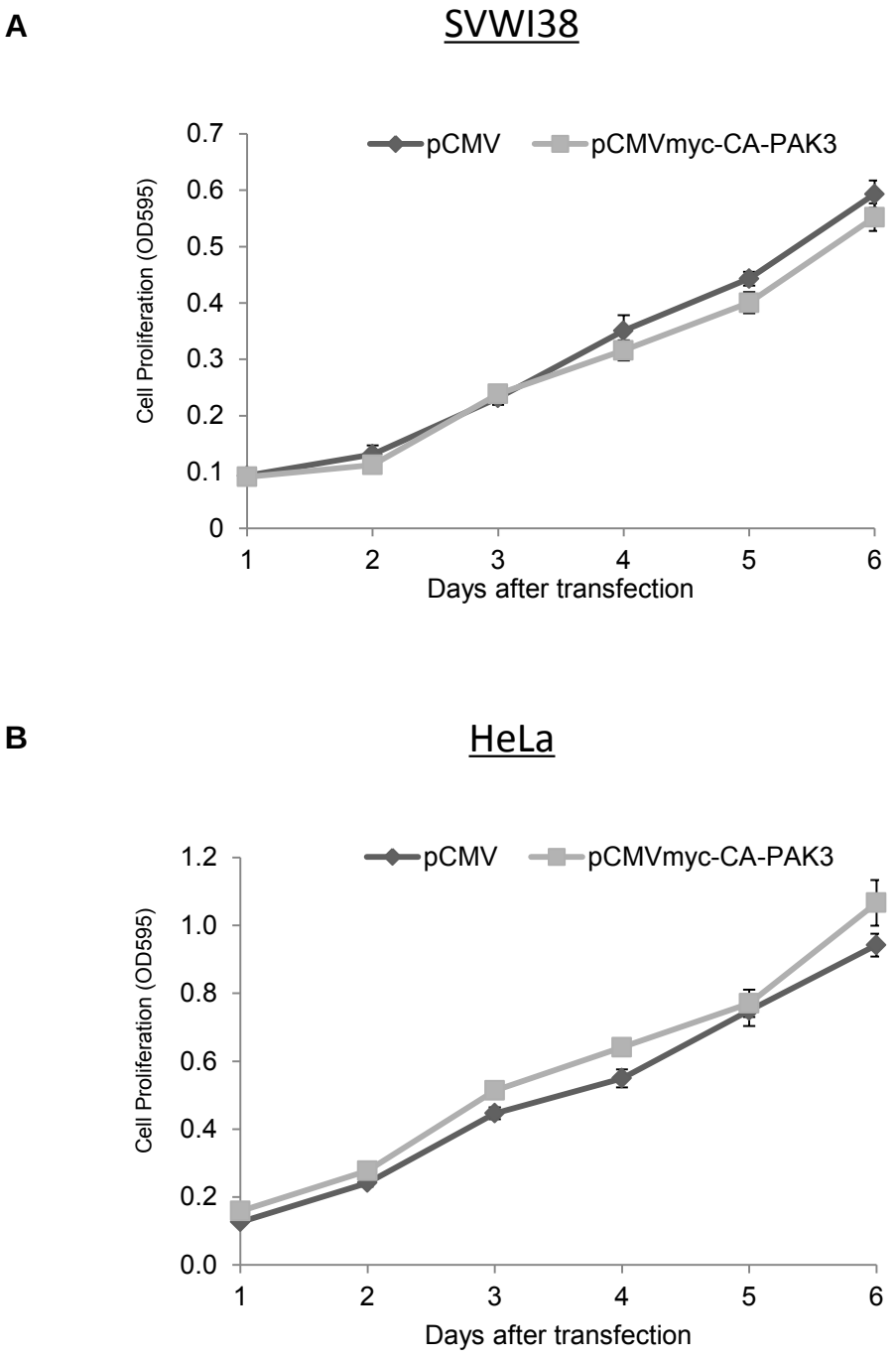


**Fig 4.3: Expression of CA PAK3 mutant has no effect on proliferation of SVWI38 or HeLa cells.** Cell proliferation assays (MTT) with SVWI38 (**A**) and HeLa (**B**) cells transiently transfected with 300 ng/ml empty vector, pCMV, or the CA PAK3 expressing vector, pCMV-myc-CA-PAK3. The results shown are the mean ± S.E. of experiments performed in six repeats and repeated twice.

appeared more pronounced in HeLa cells than SVWI38 cells. These images support our previous data that PAK3 is involved in the actin reorganization associated with transformation. This result also suggests that additional activation of PAK3, utilising the over-expression of constitutively-activated PAK3, results in even further actin reorganization in transformed and cancer cells. Furthermore, it suggests that the level and activation state of PAK3 is important for the degree of actin reorganization.

### 4.2.4. Constitutive activation of PAK3 results in changes in cJun phosphorylation

The PAK family of proteins are the primary effectors of the small GTPases, specifically CDC42 and Rac[71,80]. Once activated, the PAK proteins initiate a kinase signal cascade through the MAP kinase pathway, implementing the functional target genes associated with the specific up-stream signal. The MAP kinase pathway has many effectors, one being the phosphorylation and activation of cJun N-terminal Kinase (JNK)[126]. JNK phosphorylates cJun at two residues, serine 63 and serine 73, allowing for the dimerization of cJun and the activation of the AP-1 complex[13,21,22]. We hypothesised that, should PAK3 and AP-1 be involved in a novel feedback loop, over-expression of CA PAK3 could potentially increase levels of AP-1 phosphorylation and activation.

Western blot analysis was performed to detect total and phosphorylated cJun in response to CA PAK3. Immunodetection showed no change in total cJun protein levels in response to CA PAK3 in both SVWI38 (Fig. 4.5A) and HeLa (Fig. 4.5B) cells. However, changes in p-cJun were noticed. This response differed between cell lines. In SVWI38 cells, there was a marginal increase in the

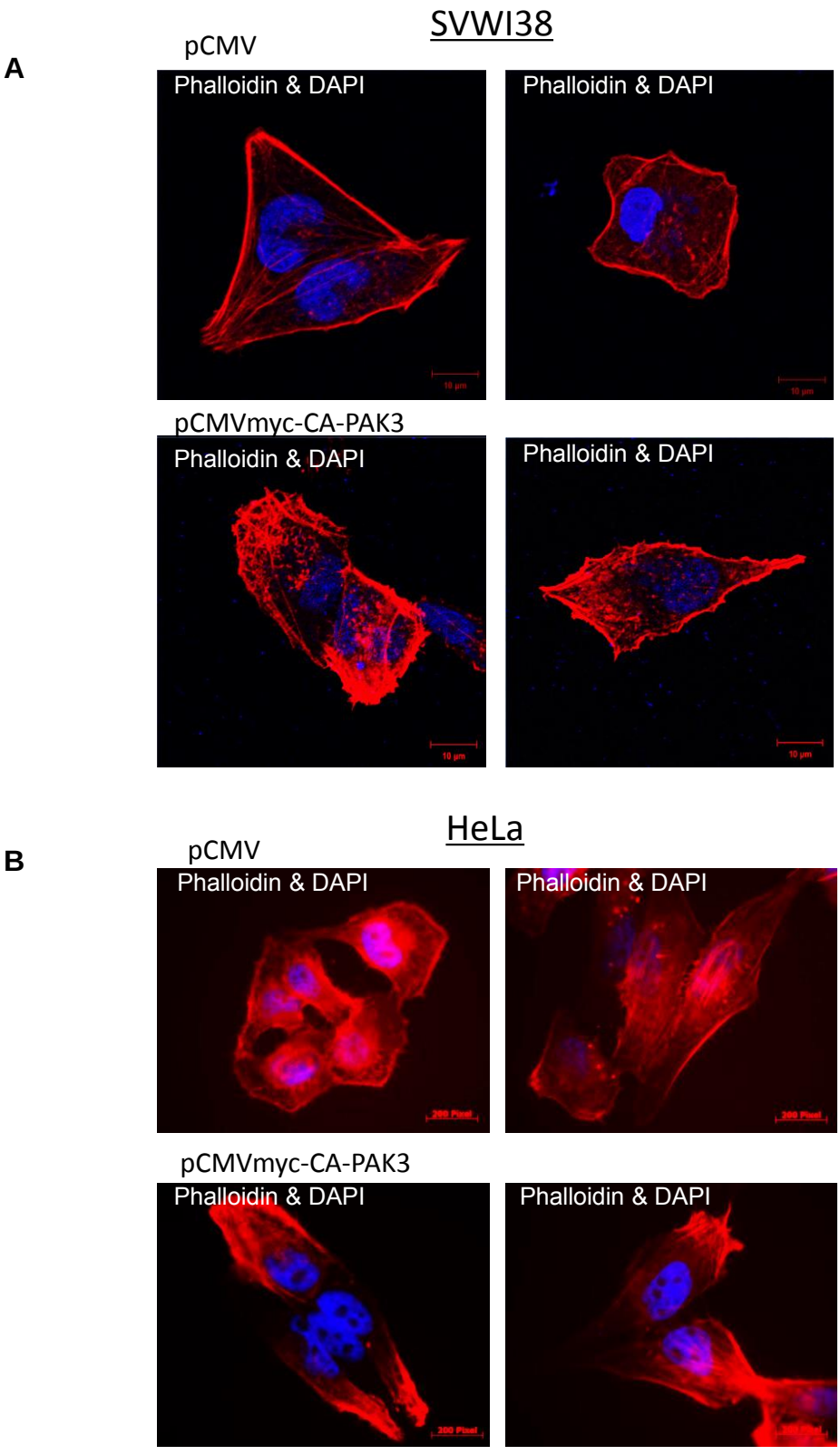


**Fig 4.4: Expression of CA PAK3 has an effect on actin reorganization in SVWI38 and HeLa cells.** Fluorescent staining of polymeric F-actin using phalloidin (red) in SVWI38 (**A**) and HeLa (**B**) cells transfected with 300 ng/ml of either the empty vector, pCMV, or the CA PAK3 expressing plasmid, pCMV-myc-CA-PAK3. Images were captured 48 hours after transfection. The staining showed actin recruitment to the ends of the cells when CA PAK3 was expressed in the cells. DAPI stain was used to visualize the cell nuclei (blue).

levels of p-cJun when using an antibody that detects phosphorylation at both serine sites (ser63/73) in response to CA PAK3 over-expression (Fig. 4.5 A). Antibodies directed against the individual phosphorylated sites of p-cJun showed a slight decrease in phosphorylation of serine 63 (ser63), and a marked increase at serine 73 (ser73) in CA PAK3 transfected SVWI38 cells (Fig. 4.5 A). HeLa cells transfected with CA PAK3 showed an increase in the levels of p-cJun (ser63/73), while p-cJun (ser73) showed no change and p-cJun (ser63) could not be successfully detected (Fig. 4.5 B).

Although the level and type of cJun phosphorylation differed between the SVWI38 and HeLa cell lines, potentially due to cell line specific variations, changes in cJun phosphorylation in response to CA PAK3 were seen in both cell lines. This is the first description that PAK3 can alter the phosphorylated state of cJun.

### 4.2.5. Constitutive activation of PAK3 results in the activation of cJun

As there were changes in cJun phosphorylation upon CA PAK3 expression, we next determined whether PAK3 activation leads to increased AP-1 activation. In order to determine the level of AP-1 activity in response to CA PAK3, we performed AP-1 reporter assays. This assay utilized the 4X-AP-1 construct, containing four AP-1 binding sites cloned upstream of a luciferase gene. When activated, AP-1 is able to bind its consensus sites resulting in expression of the luciferase gene.

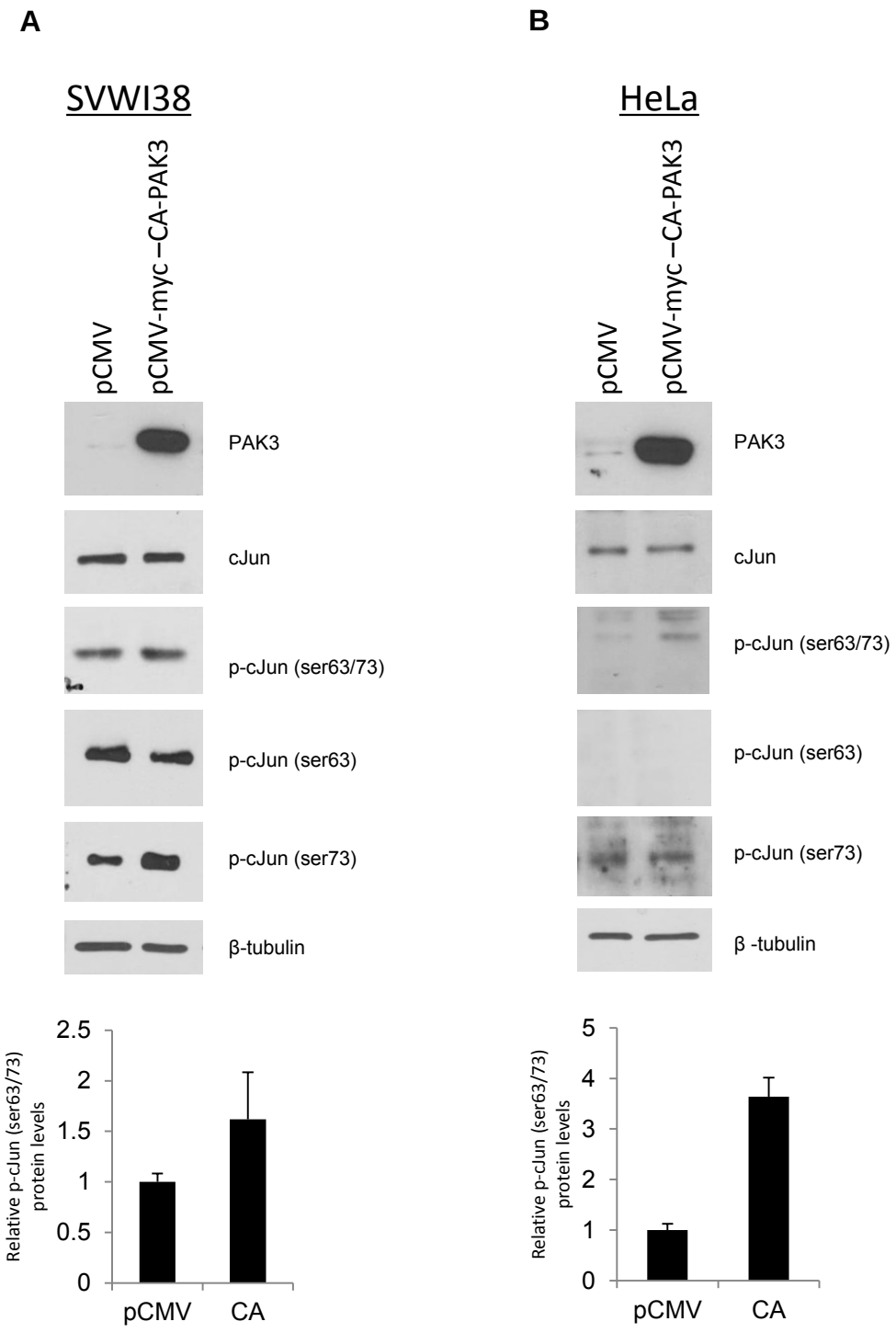


**Fig 4.5: Expression of CA PAK3 results in changes in cJun phosphorylation in SVWI38 and HeLa cells.** Western blot analysis showing the levels of PAK3, cJun and phosphorylated cJun protein in SVWI38 (**A**) and HeLa (**B**) cells transfected with 300 ng/ml of empty vector, pCMV, or CA PAK3 expressing vector, pCMV-myc-CA-PAK3. Protein was harvested 48 hours after transfection and immunodetected for total levels of PAK3 and cJun, as well as levels of phospho-cJun (p-cJun) at the most commonly phosphorylated residues, serine 63 and serine 73. Bar graphs represent p-cJun (ser63/73) protein levels, relative to the β-tubulin levels in each sample, $\pm$ S.E. over three independent experiments.

SVWI38 and HeLa cells were transfected with both the 4X-AP-1-Luc and the pCMV-myc-CA-PAK3 plasmid, and luciferase activity measured using the dual-luciferase™ reporter assay. In both cell lines, luciferase activity in response to CA PAK3 was significantly higher than that observed with the empty vector, pCMV (Fig. 4.6A and B), indicating that activated PAK3 results in AP-1 activation. This result provides evidence for a novel feed-back mechanism between the two proteins, where AP-1 induced PAK3 expression and PAK3 in turn activates AP-1.

### 4.2.6. Addition of SP600125, a JNK inhibitor, was able to inhibit the CA PAK3 induced activation of AP-1.

The PAK proteins have been shown to be activators of the JNK signaling cascade[196]. Since CA PAK3 expression results in increased AP-1 activity and changes in cJun serine 63 and 73 phosphorylation, we asked whether PAK3 was activating AP-1 through JNK, and whether the JNK inhibitor, SP600125, was capable of blocking the CA PAK3 induction of AP-1 activity. SP600125 is a low-molecular weight compound which was first discovered due to its ability to significantly inhibit JNK1, 2 and 3 as a reversible ATP-competitive inhibitor[199].

To investigate the effect SP600125 had on the increased AP-1 activity, we used promoter reporter assays with the 4XAP-1-Luc reporter plasmid in SVWI38 and HeLa cells. As a control for measuring the effects of SP600125, we used TPA, which results in the activation of the kinases that phosphorylate JNK[200], to induced AP-1 activation. We showed that the TPA-induced activation of AP-1 was significantly inhibited by SP600125 in SVWI38 and HeLa cells (Fig. 4.7A and B). To investigate the effect of SP600125 on CA PAK3-induced AP-1 activity, cells were

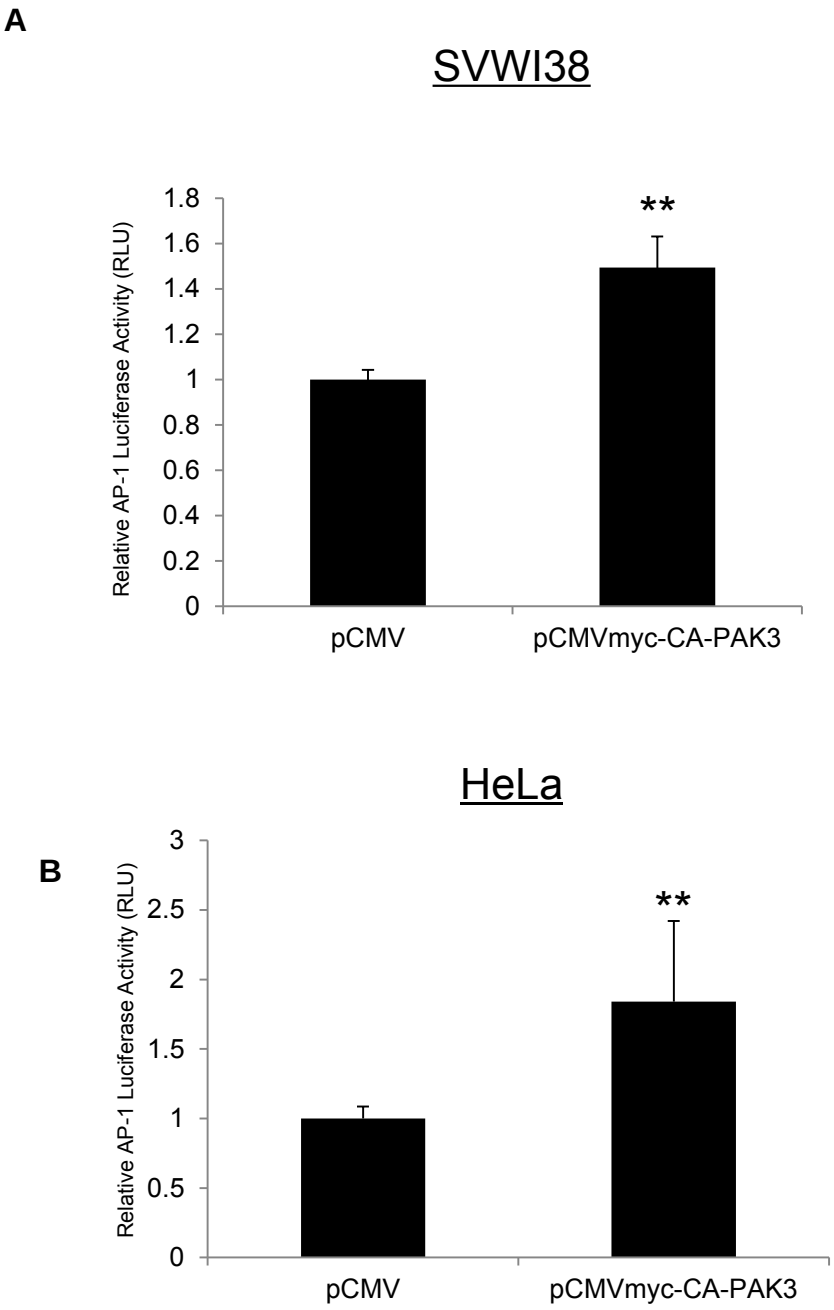


**Fig 4.6: Expression of CA PAK3 results in increased AP-1 activation in SVWI38 and HeLa cells.** Luciferase reporter assays performed with a plasmid containing four wildtype AP-1 binding sites upstream of a luciferase gene, 4XAP-1-Luc, were used to show AP-1 activation in SVWI38 (**A**) and HeLa (**B**) cells transfected with either the empty vector, pCMV, or the CA PAK3 expressing plasmid, pCMV-myc-CA-PAK3. For all reporter assays, TK-Renilla-Luciferase was used as an internal control for transfection efficiency and luciferase activity was expressed relative to Renilla luciferase in each sample. Results show the mean $\pm$ S.E. of experiments performed in quadruplicate and repeated four times. (**$p \leq 0.01$).

transfected with the 4XAP-1-luc, alongside either the pCMV or the CA PAK3 plasmid, which induced AP-1 activity. The addition of SP600125 resulted in a significant reduction in AP-1 activity in CA PAK3 transfected SVWI38 and HeLa cells (Fig. 4.7C and D).

This result suggests that PAK3 regulates AP-1 activity through JNK, the target of the SP600125 inhibitor.

### 4.2.7. Constitutive activation of PAK3 does not result in the phosphorylation of p-JNK or p-ERK

Published studies have reported that JNK1/2 are mainly responsible for the activation of AP-1 by phosphorylation of cJun on serine 63 and 73 residues[13,23]. Likewise, ERK1/2 have been shown to phosphorylate cJun on these same residues[24]. Activity of the PAK proteins has been seen to regulate both ERK and JNK/SAPK activation through the MAP Kinase pathway[196,197,201–203].

We have shown that the JNK inhibitor blocks the CA PAK3-induced activity of AP-1, suggesting that PAK3 requires the activation of JNK to activate AP-1. We, therefore, next investigated the activation of JNK in response to CA PAK3 expression by western blot analysis. Since PAK3 can also activate ERK, which is also known to phosphorylate Jun, we also assayed ERK activation. Both JNK and ERK require phosphorylation in order to become activated, hence p-JNK and p-ERK were used as a measure of their activation. Although the phosphorylation of these two proteins is transient, with the activation of the proteins dying-out once the signal has died out,

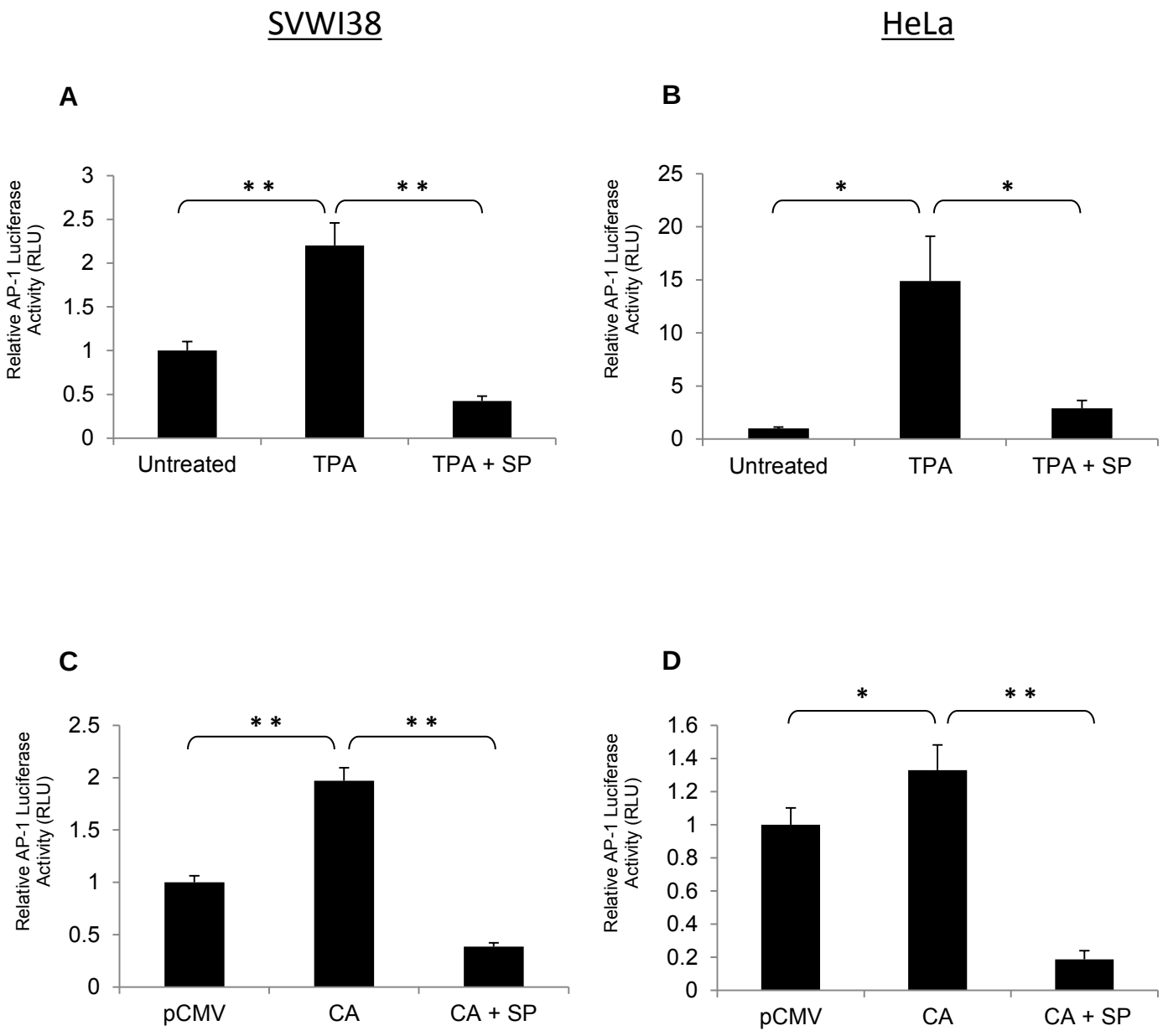


**Fig 4.7: Treatment with SP600125 reduces CA PAK3-induced AP-1 activity in SVWI38 and HeLa cells. A & B:** Luciferase reporter assays performed with the 4XAP-1-Luc plasmid, were used to show cJun activity in SVWI38 (**A**) and HeLa (**B**) cells treated with 100 nM TPA to induced AP-1 activity, then treated with 40 µm SP600125 (SP), a serine/threonine kinase inhibitor. **C & D:** Luciferase reporter assays performed with the 4XAP-1-Luc plasmid in SVWI38 (**C**) and HeLa (**D**) cells transfected with 300 ng/ml of either the empty vector, pCMV, or the CA PAK3 expressing plasmid, pCMV-myc-CA-PAK3 (CA). Cells transfected with the CA PAK3 expressing vector, were then further treated with 40 µm SP600125 (SP), and luciferase activity measured. For all promoter assays, TK-Renilla-Luciferase was used as an internal control for transfection efficiency and luciferase activity was expressed relative to Renilla luciferase in each sample. Results show the mean $\pm$ S.E. of experiments performed in quadruplicate and repeated twice. (*$p \leq 0.05$ and ** $p \leq 0.01$).

in the presence of CA PAK3 we anticipated seeing the downstream kinase(s) responsible for regulating AP-1 activity constantly activated, as CA PAK3 would be constantly signalling to them.

Western blot analysis for p-JNK 1/2/3 and p-ERK 1/2 in CA PAK3 transfected SVWI38 and HeLa cells was performed. Results showed no change in the total levels of JNK or ERK 2, as well as no change in the phosphorylation state of p-JNK or p-ERK in either SVWI38 (Fig. 4.8A) and HeLa (Fig. 4.8B) cells. This result shows that neither JNK nor ERK appear to become activated in response to CA PAK3 and thus are unlikely to be responsible for the activation of AP-1 in response to CA PAK3.

Since JNK phosphorylation did not change in response to CA PAK3 expression, this result suggests that the reduction seen in CA PAK3-induced AP-1 activity in response to SP600125, was not due to the inhibition of JNK. As ERK was also not activated in response to CA PAK3, it suggests that ERK was also not the kinase mediating PAK3 regulation on AP-1, but rather that CA PAK3 increased AP-1 activity through an alternative kinase that responds to SP600125.

Although SP600125 has widely been used as a specific JNK inhibitor, the drug has many off-target effects. As a serine/threonine kinase inhibitor, SP600125 has been shown to inhibit multiple other kinases with great efficacy[204], and recently, has been shown to inhibit a MAPK-related kinase, MAKPK interacting serine/threonine kinase 1 (MKNK1)[205]. Since our data suggests that JNK and ERK are likely not involved in the increased AP-1 activity seen in response

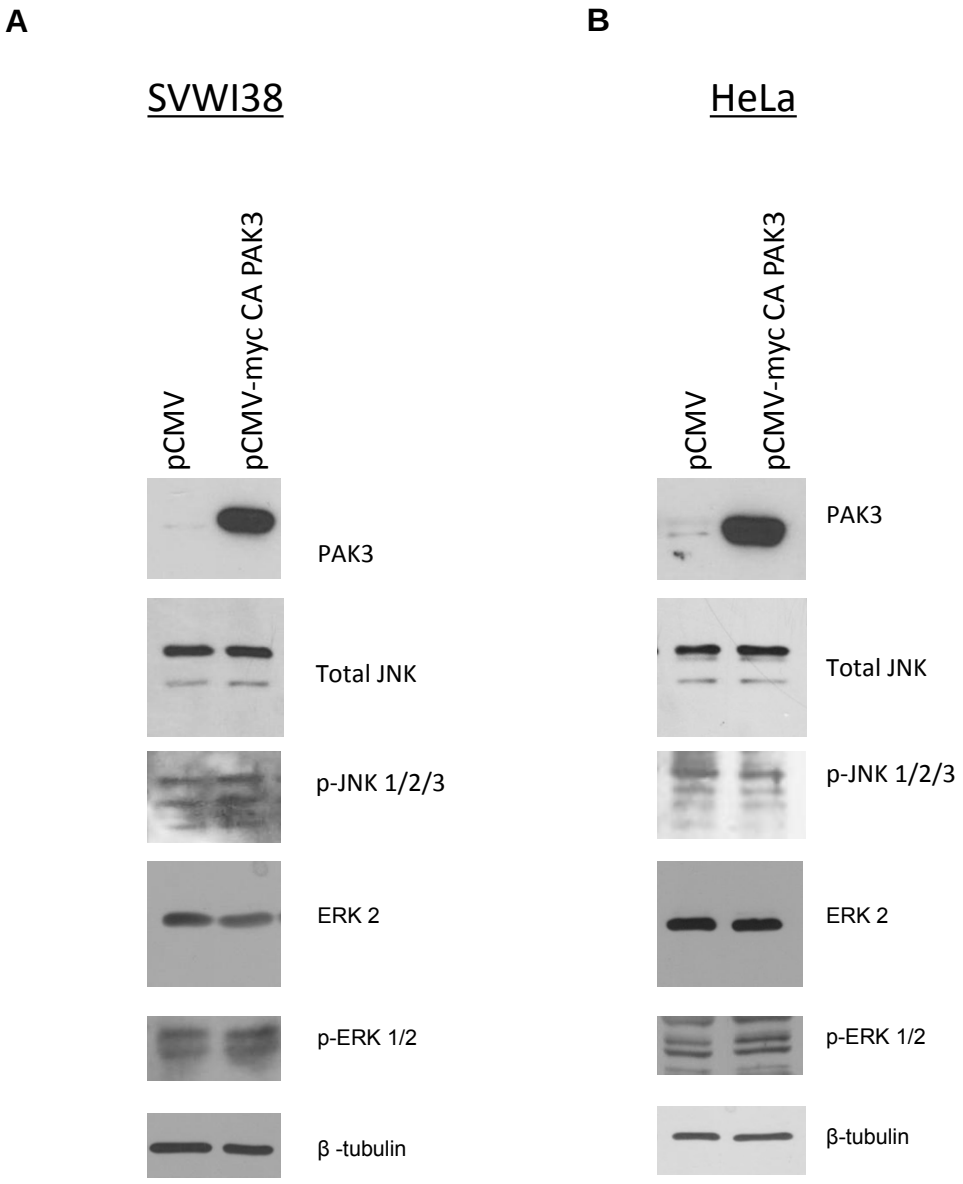


**Fig 4.8: Expression of CA PAK3 does not affect activation of JNK or ERK kinases in SVWI38 or HeLa cells.** Western blot analysis showing the level of total and phosphorylated (p-) JNK and ERK protein in response to CA PAK3 over-expression in SVWI38 (**A**) and HeLa (**B**) cells. Cells were transfected with 300 ng/ml of the empty vector, pCMV, or the CA PAK3 expressing plasmid, pCMV-myc-CA-PAK3, and protein was harvested 48 hours later. Protein was analyzed for levels of phosphorylated/activated kinases that may be responsible for the increase in AP-1 activity.

to CA PAK3, we questioned whether PAK3, as a MAP-related kinase, could be the responsible kinase.

### 4.2.8. Activated PAK3 binds cJun directly

PAK2 has been seen to increase AP-1 activity through the direct phosphorylation of cJun[191]. Having shown that neither JNK nor ERK are activated in response to CA PAK3, we next questioned whether CA PAK3 could directly be responsible for AP-1 activation. To examine this, we investigated whether there was an interaction between CA PAK3 and AP-1 using a co-immunoprecipitation technique.

Protein was extracted from SVWI38 and HeLa cells transfected with plasmids for CA PAK3 and cJun: pCMV-myc-CA-PAK3 and pCMV-cJun, respectively. The extracted proteins were cross-linked and a PAK3 antibody was used to pull-down PAK3 protein, individual or complexed. Once the cross-linking was reversed, proteins were subjected to western blot analysis for cJun and PAK3 protein detection. Pull-down assays showed that CA PAK3 and cJun interacted with each other in both SVWI38 (Fig. 4.9A) and HeLa (Fig. 4.9B) cells. A negative control with no added antibody (no Ab) confirmed that the detected cJun was not as a result of a non-specific interaction with the beads used, but rather due to an interaction with pulled-down PAK3.

While this result does not directly implicate PAK3 as the kinase responsible for AP-1 activation, the direct interaction between PAK3 and AP-1 suggests that PAK3 could be a contender as the serine/threonine kinase responsible for CA PAK3-induced AP-1 activation. Although further

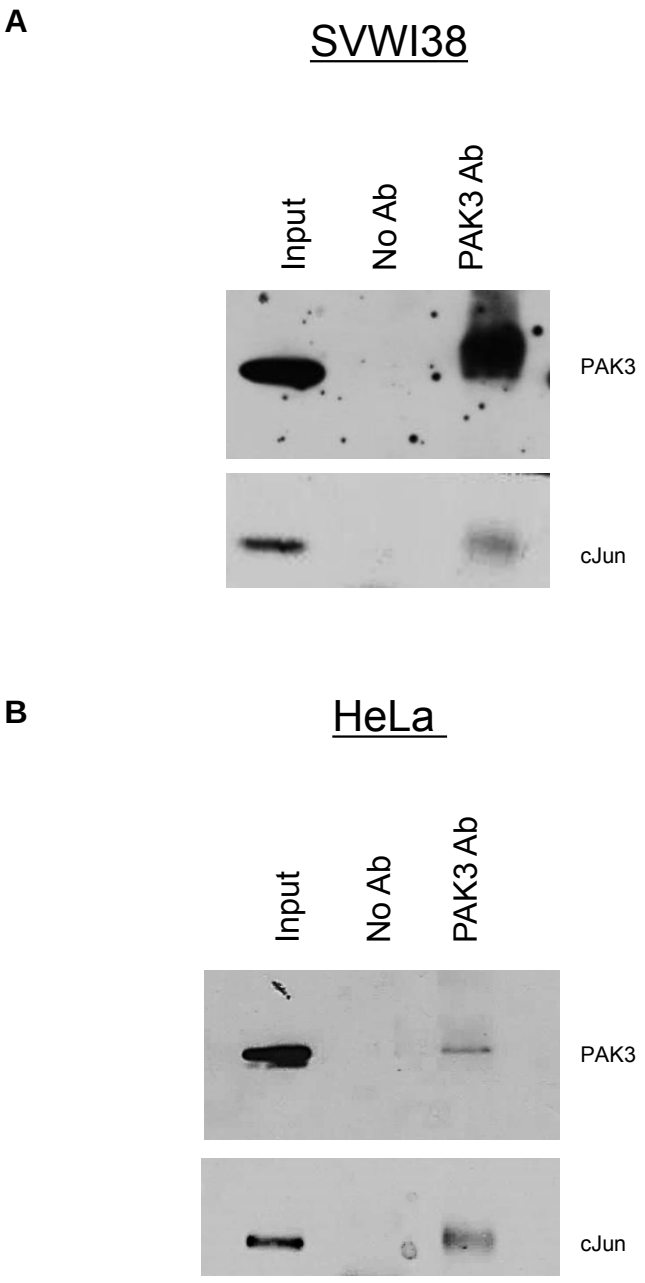


**Fig 4.9: PAK3 interacts directly with cJun in SVWI38 and HeLa cells.** Co-immunoprecipitation assay utilizing pull-down with a PAK3 antibody, showed that PAK3 interacts directly with cJun in SVWI38 (**A**) and HeLa cells (**B**). Cells were co-transfected with 300 ng/ml of the CA PAK3 plasmid, pCMV-myc-CA-PAK3, and 150 ng/ml of a cJun expressing plasmid, pCMV-cJun, and protein was harvested in a non-denaturing buffer 48 hours later (Input – protein prior to pull-down). Protein complexes were cross-linked and pulled-down with a PAK3 antibody (PAK3 Ab). Pulled down protein complex were then denatured, electrophoresed and immunodetected for the presence of cJun and PAK3. cJun protein could be detected in both the Input protein and in the PAK3 pulled-down protein. The no antibody control contained no cJun or PAK3 protein, confirming the specificity of the interaction.

experiments will need to be done to investigate this possibility, this is a novel finding suggesting that cJun might be targeted for phosphorylation by PAK3.

Taken together, these results show that PAK3 and AP-1 appear to have novel interactions, as AP-1 activates PAK3 expression, and over-expression of constitutively-activated PAK3 increases AP-1 activity. JNK and ERK are not involved in this feedback loop, while a target of the serine/threonine kinase inhibitor, SP600125, is vital. PAK3 and cJun directly interact with one another; however, the effect of this interaction is still to be discovered.

## 4.3. DISCUSSION

The diverse and dynamic cellular actions of proteins are controlled mainly by post-transcriptional modifications – such as phosphorylation[206]. These modifications allow proteins to be involved in molecular interactions[207], alter their sub-cellular localization[208] and control the transcriptional regulation of other genes[209].

With a cancer cell gaining the ability to generate its own signals, becoming self-sufficient from the external prompting needed to generate cellular actions[4], an avenue of perpetuating such signals is through the generation of a feedback loop between post-transcriptional modifications and transcriptional regulation of a protein. In this chapter, we investigated whether PAK3 and AP-1 exist in such a feedback loop in transformed and cancer cells.

We have shown that the expression of PAK3 with an activating mutation results in further actin reorganization in SVWI38 and HeLa cells. These changes in actin distribution, where the polymerized actin is recruited to the opposite ends of transformed cells, suggest a polarization of the cell. This is in keeping with previous work performed using the yeast homologues of the PAK family, where PAK proteins were seen to regulate cell and actin polarization at several points in the cell cycle[210]. When these PAK homologues were inactivated, there was a loss of cytoskeletal polarity but not the de-polymerisation of F-actin, suggesting that PAK proteins are involved in the redistribution of actin rather than the polymerisation. The polarisation of actin is directly linked to the motility of the cell: polarisation of the actin cytoskeleton, by the Hippo

pathway, is needed for collective migration in Drosophila border cells[211], while actin was found to be polarised in an area of high motility in T lymphocytes[212]. Along with our previous data showing the involvement of PAK3 in the motility of transformed and cancer cells, the polarization of constitutively-activated (CA) PAK3 expressing cells is further evidence for PAK3's role in migration. The transcription factor AP-1 has also been highly implicated in the increased motility of transformed cells[50,54]. This would suggest a potential dual function for PAK3 and AP-1 in migration. We have shown that this potential dual role is perhaps a shared role. Previously, we showed that PAK3 inhibition in cJun/AP-1 over-expressing cells resulted in a significant decrease in the motility of transformed cells, where PAK3 was a direct target of AP-1. In this chapter we have shown that activation of PAK3 results in changes in AP-1 phosphorylation and an increase in its activity. Taken together, this would suggest a novel feedback loop between the two proteins, where AP-1 transcriptionally activates PAK3, which in turn activates AP-1.

There is evidence to suggest AP-1 in other feedback loops. Another AP-1 target, sufiredoxin, an enzyme that regulates reactive oxygen species signaling by reducing hyperoxidized peroxiredoxins[213,214], is thought to contribute to the activation of AP-1[69]. Sulfiredoxin is a transcriptional target of AP-1 which is required for the transformation of JB6 cells[69] and depletion of sulfiredoxin signaling impaired AP-1 activation and increased cell death under oxidative stress conditions. This positive feedback loop is thought to be achieved, in part, through the regulation of JNK1/2 kinase activity[69].

To our knowledge, PAK3 has not yet been implicated in a signaling loop. We have confirmed that PAK3 is a target gene of AP-1 and although there is no direct evidence in literature to suggest that PAK3 regulates AP-1, there is evidence to suggest that the PAK family of proteins lie upstream of AP-1 activation. PAK3 has been shown to activate the JNK signaling cascade in response to CDC42 activation[196]. The inhibition of either PAK1 or MEKK1, which are thought to function independently of one another, prevents JNK/AP-1 activation[12,90,197]. This evidence suggests that PAK3, along with the other PAK proteins, lie upstream of the signaling pathway that activates AP-1. Although PAK activity has been seen to regulate ERK and JNK/SAPK[196,197,201–203], our data has shown that neither JNK nor ERK appear to be activated in response to constitutively-activated PAK3.

Barbiturates, a class of drugs derived from barbituric acid which are known to suppress protective immunity, have been shown to affect the MAP kinase pathway by inhibiting the small G proteins, Ras and Rac1, as well as by binding to Raf1, or the PAK proteins[215]. Their ability to bind PAK proteins in the brain has allowed them to be used to treat patients with severe brain injury[216]. The barbiturates can also block AP-1 dependent gene expression in T lymphocytes independently of the small G proteins and the MAP Kinase pathway[215]. A specific barbiturate, thiopental, inhibits AP-1 but does not directly affect the p21ras/MAP pathway[215]. Together, work done to understand barbiturate signaling suggests an additional unknown mechanism of AP-1 regulation, other than through the MAP Kinase pathway.

In a genome-wide microarray of human splenic marginal zone lymphomas, a number of the AP-1 family members, namely cJun, JunB, JunD and cFos, were seen to be up-regulated[51]. However, this microarray did not show high expression of the MAP kinase pathway: the signalling cascade that is traditionally thought to activate AP-1. This further suggests AP-1 activation by an unidentified kinase(s).

There are over 500 separate kinase genes encoded in the mammalian genome[25], making protein kinases one of the most abundant eukaryotic regulatory proteins[217,218]. PAK2 has been shown to bind and phosphorylate cJun on five threonine residues[191]. Although PAK2 played no role in changing the phosphorylation state of the Jun serine 63 and 73 residues, phosphorylation of the five threonine residues was directly linked to the transformation ability of AP-1[191]. However, PAK2 was not identified to be a target gene of AP-1 in our study as it <sup>70</sup><sup>70</sup><sup>69</sup><sup>68</sup><sup>68</sup>did not respond to doxycycline induction of cJun/AP-1 over-expression.

PAK3, which we have shown to be a transcriptional target of AP-1, appears to also interact directly with cJun. We showed in co-immunoprecipitation assays that PAK3 and AP-1 complex together in both SVWI38 and HeLa cell lines, suggesting the possibility of a regulatory mechanism between them. In order to investigate this regulation, we used SP600125. SP600125 is a low-molecular-weight compound that has been widely used to inhibit c-Jun-N-terminal kinase (JNK); however there are many off target effects. Bain et al. (2003)[204] showed that SP600125 inhibited 13 out of 28 kinases tested with a potency as great as its activity

against JNK. Of these *kinases,* SGK, p70 ribosomal protein S6 kinase (S6K1), AMPK, CDK2, CK1d and DYRK1A were all inhibited to a greater extent than JNK[204]. None of the PAK kinases were tested by Bain et al. (2003)[204]. An independent study showed that PI3K has also been seen to be inhibited by SP600125[219]. The off-targets effect of SP600125 lead to the recognition of a serine/threonine MAPK-related kinase involved in the viral entry and replication of the hepatitis C virus[205]. Since SP600125 has been seen to dose-dependently inhibit the phosphorylation of cJun[199], we asked whether SP600125 could reduce the increased AP-1 activity observed in the presence of activated PAK3, a serine/threonine kinase.

We showed that SP600125 was capable of inhibiting the activation of AP-1 in the presence of CA PAK3. Although this result does not answer if PAK3 is the kinase responsible for the increase in AP-1 activity, it does provide further evidence for an as yet unidentified serine/threonine kinase, other than JNK or ERK, capable of activating AP-1. Further PAK3 kinase assays would have to be performed to elucidate whether PAK3 is the serine/threonine kinase responsible for the changes in cJun phosphorylation and activation of AP-1.

Taken together, our results are a first to show that PAK3 activation results in an increase in AP-1 activity and implicate PAK3 and AP-1 within a positive feedback loop in which JNK and ERK do not appear to be involved.

# CHAPTER 5

# CONCLUSIONS

The development of cancer is complex, with multiple insults and genes playing a role in the process. Ultimately, transcription factors participate at the ends of many oncogenic pathways, perpetuating deregulated signals and causing the up- or down-regulation of specific genes that change the transcriptional pattern of the cell[59,220]. The Activating Protein 1 (AP-1) is one such transcription factor; its deregulation is required for various oncogenic signals[18,41] and its over-expression has been linked to many cancers[42–47]. Although AP-1 is highly implicated in cancer, little is known about which of its target genes are essential for implementing its oncogenic function[10]. Identifying and understanding such targets will not only allow for a deeper understanding of the molecular events involved in the transformation of a normal cell to a cancer cell, but will provide more options in typing and combating the disease.

This study stems from previous work done to identify the genetic events that occurred during the transformation of rat fibroblasts through the de-regulated expression of AP-1[9,70]. The study identified p21-Activated Kinase 3 (PAK3) to be up-regulated in response to cJun/AP-1 over-expression and predicted PAK3 to be an AP-1 target gene involved in transformation. In this study, we have confirmed that PAK3 is an AP-1 target gene, and that AP-1 controls the expression of PAK3 through direct binding to a single AP-1 binding site in the PAK3 promoter.

We have shown that PAK3 is a direct transcription target of AP-1. To our knowledge, this is the first description of PAK3 as an AP-1 target gene and the first study to implicate a p21-Activated Kinase as an AP-1 regulated gene[221].

With AP-1 playing a role in transformation and a number of different cancers, we investigated the levels of PAK3 in transformed and cancer cell lines. PAK3 expression was seen to be consistently elevated in both rat and human transformed fibroblasts and in an array of cancer cell lines from different tissues origins. These cell lines were derived from the gynaecological cancers of the cervix, ovary and breast. Previously PAK3 expression was thought to be relatively restricted to the neurons[71]; however, our data suggest that PAK3 has a much wider range of expression than was previously thought. This increased range of expression is supported by data that has shown elevated PAK3 expression in the cervical cancer cell line, HeLa[133], and PAK3 mutations in lung adenocarcinomas[132]. The other Group I PAK members, PAK1 and PAK2, are known to have a wide range of expression. PAK1 is highly expressed in the brain, muscle and spleen[71], while its over-expression has been seen in ovarian, breast, bladder and lymph cancers[115]. PAK2 is thought to be ubiquitously expressed in tissues and its elevated expression has been observed in prostate, ovarian and breast cancer[115,119,120].

A member of the Group II PAK proteins, PAK4, is known to be elevated in numerous cancer cell lines[121]. With further investigation into the role and expression of PAK3, it is likely that, similarly to the other PAK proteins, its expression in cancers may prove to be even more wide-reaching. In investigating PAK3 expression in cancers, we have shown that PAK3 expression was negligible

in the non-transformed and "normal" cell lines we assayed, compared to the transformed and cancer cell lines, suggesting a role for PAK3 in the transformed phenotype.

Utilizing inhibition of the elevated PAK3 expression alongside expression of constitutively-activated PAK3, we have identified a role for PAK3 in the transformed phenotype. Elevated PAK3 expression was shown to be directly linked to the morphology and motility of rat and human transformed fibroblasts, as well as a cervical and ovarian cancer cell line. Additionally, elevated PAK3 expression resulted in a re-distribution of actin in these cells, causing cellular polarisation and increasing the cytoplasmic protrusions from the cells. The actin cytoskeleton of a cell plays a major role in the cellular morphology and polarity, as well as the assembly of filamentous actin structures in the cell[222]. These changes in the cytoskeleton provide a driving force for processes such as motility. The PAK family of proteins are the effectors of the Rho family of GTPases[71,80], which are known to be the regulators of actin reorganization[170]. Therefore, we predict that the changes seen in actin distribution are the primary effects of increased PAK3, which in turns lead to changes in morphology and increase motility. Although PAK3 phosphorylation of Raf1 has been shown to cause anchorage independent growth[105] – a characteristic of transformation - PAK3 has never directly been implicated in transformed phenotype. Our results are a first to implicate PAK3 in the transformed phenotype of morphology, actin reorganization and migration.

Although we showed that activated PAK3 had a functional role in morphology and motility of transformation, our data suggest that PAK3 does not play a role in increased proliferation of

transformed and cancer cell lines. PAK1 has been shown to play a role in proliferation, in promoting cell survival, however these effects are often indirect[223], or seen in conjunction with other proteins. For example, PAK1 inhibition alone, resulted in delayed cell-cycle progression, while the simultaneous inhibition of PAK1 and the X-chromosome-linked inhibitor of apoptosis efficiently increased apoptosis in a lung carcinoma[224]. Similarly, inhibition of PAK1 and cyclin D1 by curcumin reduced proliferation in human gastric cells[225]. PAK2 is fundamentally different from the other PAKs, in that it plays a dual role in apoptosis. Normally, PAK2 behaves as PAK1 does, promoting cell survival through similar mechanisms[226]. However, when PAK2 is cleaved by caspase-3, a truncated version of the protein accumulates at the plasma membrane and promotes cell death[97]. Inhibition of the individual Group I PAK proteins was found to be insufficient to inhibit increased proliferation of neurofibromatosis type 2; however simultaneous inhibition of all three PAK proteins successfully decreased proliferation[188].

Thus, although our results suggests that PAK3, like PAK1, does not have a direct role in proliferation, further inhibition of PAK3 alongside inhibition the other Group I PAK proteins would provide a clearer understanding of the role PAK proteins play in the proliferation of transformed and cancer cells. However, the role of PAK3 in morphology and motility, but not proliferation, specifically in an ovarian cancer cell line, is supported by Siu *et al.* (2010)[120]. They showed that knock-down of PAK1 and PAK2 in ovarian cancer cell lines reduced migration, but did not affect the proliferation of the cells.

PAK3 expression, although seen to be elevated in an array of cervical and ovarian cancer cell lines, did not correlate well with cancer patient material. In cervical cancer patient material, a trend towards an increase in PAK3 expression was observed, whereas in serous ovarian cancer material we found PAK3 expression to be significantly decreased in cancer samples compared with the normal tissue. Although our samples sizes were relatively small (cervical: normal (n=6), cancer (n=15); ovarian: normal (n=5), cancer (n=49)), there is a study which suggests that PAK3 expression is silenced in cancers. Cancer-specific methylation of the PAK3 gene was found at a high frequency in oesophageal, lung, cervical, bladder and head and neck cancers, while methylation of PAK3 was only occasional in normal tissues[134]. Having access to DNA copy number data of 72 serous ovarian cancer patients, we looked at the number of PAK3 copies in these samples. We found that the X-chromosome, along with the PAK3 gene, was deleted with a high frequency. Over 50% of patients showed a deletion of the gene and the X-chromosome, while only approximately 14% showed a DNA amplification of the PAK3 gene. These deletions could explain the low expression levels of PAK3 seen in the assayed ovarian cancer patient material. Interestingly, we showed that serous ovarian cancer patients with high PAK3 expression had a significantly poorer survival than those with low PAK3 expression. With PAK3 playing a role in the motility of transformed and cancer cell lines, it could be possible that the poor survival associated with high PAK3 expression is linked to the aggression of the disease. There is evidence in the literature that elevated expression of both PAK1 and PAK2 associates with the poor over-all survival of ovarian cancer patients[120]. Therefore, PAK3, in addition to PAK1 and PAK2, may serve as a useful prognostic marker, especially in serous ovarian cancer.

PAK3's link to the poor survival of serous ovarian cancer patients may additionally be as a result of self-sufficiency in signals and an independence from external promptings for cellular function. In this study, we provide evidence suggesting that PAK3 and AP-1 are partners in a novel, positive feedback loop. Research to date places the PAK family of proteins upstream of the activation of AP-1[12,19,196], while PAK2 has been shown to directly phosphorylate AP-1, and this phosphorylation is required for the transforming ability of AP-1[191]. We found that PAK2 expression was not responsive to AP-1 induction, while PAK3 expression was. We have also shown that activation of PAK3 results in changes in cJun phosphorylation and an increase in AP-1 activity. Activation of PAK3 did not result in the activation of JNK or ERK, the kinases known to activate AP-1 through cJun phosphorylation[13,23,24]. However, increased AP-1 activity in response to activated PAK3 could be inhibited by the addition of the JNK inhibitor, SP600125. SP600125 has been shown to have many off-target effects, inhibiting a large number of kinases with equal or greater efficiency than JNK[204]. Since SP600125 is a serine/threonine kinase inhibitor, PAK3 could be a potential candidate as the kinase responsible for the activation AP-1. In support of this, we have shown that PAK3 and AP-1 proteins directly interact with one another. However, we cannot say what the effect of this interaction on cJun/AP-1 is, as yet. Further investigations, such as PAK3 kinase assays, could elicit a better understanding of the novel relationship between PAK3 and AP-1.

In summary, we have shown that PAK3 is an AP-1 target gene directly activated by the binding of AP-1 to a single site in the PAK3 promoter. This activation of PAK3, in turn, activates AP-1 through a novel feedback loop. PAK3 expression is elevated in rat and human transformed

fibroblasts and in an array of cancer cell lines from different origins. The increased expression of PAK3 in these cells plays a role in the morphology, actin reorganization and motility. Due to various adaptations of *in vivo* cancers, elevated PAK3 expression does not correlate with tumours; however plays a significant role in the survival of ovarian cancer patients, suggesting that the Group I PAK proteins may have use as prognostic markers for ovarian cancer.

# CHAPTER 6

# MATERIALS AND METHODS

## 6.1. MATERIALS

### 6.1.1. Cell lines

Origins

Rat model system cell lines, Rat1a-J4 and Rat1a-GFP, were obtained from Dr Howard Donninger (Louisville University, USA). The experimental cell line, Rat1a-J4, contains a doxycycline-inducible cJun construct, while the control cell line, Rat1a-GFP, contains a doxycycline-inducible green fluorescent protein (GFP) construct. Both cell lines were maintained by selection with 5 µg/ml blastacidin (Invitrogen, Life Technologies, Carlsbad, CA, USA) or induced for gene expression with 2 µg/ml doxycycline (Sigma-Aldrich, St. Louis, MO, USA).

The below human cell lines were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA):

- The normal lung fibroblast cell line, WI38, and its SV40 transformed counterpart cell line, SVWI38
- The cervical carcinoma cell lines: CaSki, HeLa, ME180, SiHa, MS751 (all HPV-positive) and C33A (HPV-negative)
- The breast carcinoma cell lines: MCF7 and MDA-MB-231, and their non-tumourigenic breast cell line counterpart: MCF12A.

The normal primary cervical epithelial cell line, HCX[227], was obtained from Dr C. Barker (NIH, USA). All ovarian cancer cell lines were provided by Prof M. Birrer (Harvard Medical School, MA, USA), who originally obtained them as follows:

- A224 were a gift from Dr J. De Greve, Oncologisch Centrum, Brussels, Belgium
- SKOV3 were a gift from Dr G. Scott Rose, University of California, Irvine
- OVCAR433 were obtained from the Laboratory of Gynecologic Oncology at Brigham and Women's Hospital, Boston, MA, USA
- OVCAR3 and UC107 cell lines were purchased from the ATCC

The normal hTERT-immortalised human oesophageal keratinocytes, EPC2 cells, were a gift from Prof A. K. Rustgi (University of Pennsylvania, Philadelphia, USA). The oesophageal carcinoma cell line WHCO1, which was derived from a South African patient with oesophageal squamous cell carcinoma, was a gift from Dr R Veale[228]. Japanese-derived oesophageal carcinoma cell lines, KYSE30, 70, 150, 180 and 450[229], were acquired from DSMZ (Berlin, Germany).

Growth media

Most cells, unless alternatively described below, were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% heat inactivated Fetal Calf Serum (FCS) (Gibco, Life Technologies, Carlsbad, CA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin. Alternatively, the non-tumourigenic breast cell line, MCF12A, was maintained in media consisting of 50% Ham's F12 media (Gibco) and 50% DMEM media (with 10% heat inactivated Fetal Calf Serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin) with 20 ng/ml EGF (Gibco), 100 ng/ml Cholera toxin (Sigma-Aldrich), 500 ng/ml Hydrocortisone (Sigma-Aldrich) and 10 µg/ml Insulin (Gibco). All ovarian cancer cell lines were maintained in RPMI-1640 medium (Gibco) with 10% fetal calf

serum (FCS), 100 U/ml penicillin, and 100 mg/ml streptomycin. The primary cervical epithelial cell line, HCX, was grown in Keratinocyte Serum Free Medium (KSFM) (Invitrogen) supplemented with 50 µg/ml pituitary extract (Gibco) and 10 ng/ml EGF (Gibco), along with 100 U/ml penicillin and 100 µg/ml streptomycin. Normal hTERT-immortilised oesophageal EPC2 cells were also grown in KSFM with 50 µg/ml pituitary extract with 100 U/ml penicillin and 100 µg/ml streptomycin, but were only supplemented with 1 ng/ml EGF.

Anchorage-dependant growth conditions allowed cells to adhere directly to the cell culture dish, while anchorage-independent conditions were mimicked by plating cells onto Poly(2-hydroxyethyl methacrylate) (Poly-HEME) (Sigma-Aldrich) -coated culture dishes. In anchorage-independent proliferation assays, cells were plated in 1% methylcelluose (Sigma-Aldrich), instead of normal media, onto poly-HEME coated dishes. Clones with stable PAK3 knock-down were maintained in an appropriate concentration of puromycin determined by puromycin cell viability assays (see 6.2.26) for each cell line.

Growth conditions

Cells were maintained at 37°C in a 5% $CO_2$ incubator and grown to 60-80% confluency before being trypsinised with 0.05% trypsin and EDTA. To freeze stocks of cells, the cells were re-suspended in media with 100 U/ml penicillin and 100 µg/ml streptomycin, 20% FCS and 10% DMSO, and frozen at -80°C for a week before being place in liquid nitrogen. More sensitive cell lines, such as WI38 cells, were thawed into media containing 20% FCS for the first 24 hours. Less sensitive cells were thawed into their normal growth media.

### 6.1.2. Patient material

All cervical patient material had previously been collected from Groote Schuur Hospital, Cape Town, South Africa and the RNA isolated by the Transcriptional Regulation and Cancer Biology Group, Medical Biochemistry, UCT, SA. The cancerous biopsies were collected from individuals being treated for late stage cervical carcinoma or dysplasia, while the normal (non-cancerous) samples were collected from patients undergoing hysterectomies for reasons other than cervical abnormalities. The patients ranged from 29 to 76 years of age. All samples were collected with the consent of the patient under the approval of the Research Ethics Committee of the University of Cape Town (REC REF153/2004) and the status of the biopsies were confirmed by a pathologist.

The ovarian cancer specimens used to perform the RT-PCR gene expression and survival analysis of PAK3 were obtained from RNA previously isolated by The Laboratory of Gynecologic Cancer Research, Harvard Medical School, USA. The RNA was from 53 snap-frozen tissue specimens from previously untreated ovarian cancer patients, who were hospitalised at the Brigham and Women's Hospital, Boston, MA, USA between 1990 and 2000. All patients had advanced stage, high-grade serous ovarian cancer according to the International Federation of Gynecology and Obstetrics (FIGO) standards. All specimens were subjected to laser-based microdissection before the RNA was isolated by. The expression profiling data by Mok *et al*. (2009)[230] was used to perform the bioinformatics analysis for the Kaplan-Meier survival curve. The DNA copy number analysis was performed on provided data of the Engler *et al.* (2012)[231] study. The study included tumour samples from 72 patients identified within prospectively collected MGH Gynecological Tissue Repository and Cedars-Sinai Women's Cancer Research

Program Tissue Bank at Massachusetts General Hospital and Cedars-Sinai Medical Center from 1991 to 2008. All samples were reviewed by a pathologist to confirm the presence of viable tumour cells in the tissue sample. These studies were reviewed by the Institutional Review Board of MGH, and informed patient consent was obtained.

### 6.1.3. siRNA

Transient gene expression knock-down was performed using short-interfering RNA (siRNA) technology. PAK3 siRNA (βPAK sc-36182, Santa Cruz Biotechnology, Dallas, TX, USA) and cJun siRNA (sc-29223, Santa Cruz Biotechnology) –mediated knock-down was performed using 20 nM siRNA with a 3:1 ratio of TransFectin Lipid Reagent (BioRad) to siRNA. Control-A siRNA (sc-37007, Santa Cruz Biotechnology) was used to control for the effect of DNA transfection. siRNAs were acquired as lyophilised powders and were suspended in RNAse-free water to give a stock concentration of 10 µM.

### 6.1.4. Drugs

Blasticidin was obtained from Invitrogen and Puromycin from Calbiochem (Merck, Whitehouse Station, NJ, USA), while doxycycline and ampicillin were both bought from Sigma-Aldrich. All of these selection markers were dissolved in sterile $H_20$, filter sterilised, aliquoted and stored at -20°C. 12-o-tetradecanoylphorbol-13-acetate (TPA) was purchased from Sigma-Aldrich (Sigma-Aldrich) and the JNK inhibitor (SP600125) purchased from Calbiochem. Both drugs were re-suspended in tissue culture grade DMSO (Sigma-Aldrich) and stored at -80°C.

### 6.1.5. Plasmids

The pGEM-T Easy plasmid (Promega, Madison, WI, USA) was used to sub-clone the PCR products of the PAK3 promoter. The $pGL_3$-Basic reporter plasmid (Promega) was used to clone the finalised PAK3 promoter constructs. A plasmid encoding the Renilla luciferase reporter gene, pRL-TK (Promega), was used as an internal control for the transfection efficiency of promoter assays. As an alternative method to doxycycline-induced cJun overexpression, the pCMV-cJun plasmid[66] (which was a gift from Prof M. Birrer, Harvard Medical School, MA, USA) was used to ectopically over-express cJun. The pCMV empty vector (received from Prof M. Birrer) was used in conjunction with the transfection of pCMV-cJun to compare the effect of the plasmid transfection. An AP-1 reporter plasmid, 4XAP-1-Luc, was also kindly received from Prof M. Birrer, and was used to determine the transcriptional activity of AP-1. This plasmid contained four wildtype AP-1 binding sites (TGAC/GTCA) upstream of the minimal promoter sequence from the albumin gene and the firefly luciferase reporter gene[232]. The wildtype 4XAP-1-Luc plasmid was used in conjunction with 4X-Mut-AP-1 plasmid (received from Prof M. Birrer), in which the four AP-1 binding sites upstream of the promoter and luciferase gene had been mutated.

The PAK3 shRNA (OriGene, Rockville, MD, USA) was comprised a set of 4 plasmids, containing 4 unique 29mer gene-specific shRNA constructs in the pGFP-V-RS plasmid. Transfection with this PAK3 shRNA was performed alongside a transfection with the negative control shRNA pGFP-V-RS plasmid (empty vector) (OriGene) and the scrambled negative control non-effective shRNA cassette in the pGFP-V-RS plasmid (scrambled shRNA vector) (Origene).

The pCMV-myc-CA-PAK3 plasmid was kindly received from Dr Alan Horwitz (University of Virginia, USA)[198]. The pCMV vector was used in conjunction with this vector as a negative control.

## 6.2. METHODS

### 6.2.1. Anchorage-independent proliferation

Anchorage-independent conditions were mimicked by plating cells onto Poly(2-0hydroxyethyl methacrylate) (Poly-HEME) (Sigma-Aldrich) coated culture dishes. A plate-specific volume (Table 6.1) of 12 mg/ml Poly-HEME was poured into culture dishes and swirled across the base and sides to ensure all areas of the dish, where cells could adhere, were covered. The coated dish, with the lid ajar, was left to dry overnight in the tissue-culture hood under UV light.

Anchorage-independent proliferation assays utilised 1% methylcellulose (Sigma-Aldrich) as the growth media. Cells prepared for the assay were re-suspended in methylcellulose and plated into the Poly-HEME coated dish. The high density of the methylcellulose ensured individually plated cells did not adhere to other individual cells, measuring the viability of an individual cell to grow into a colony over the time period of the assay.

Table 6.1: Volumes of Poly-HEME used to coat cell culture dishes

| **Cell Culture Vessel** | | **Growth Area ($cm^2$)** | **Poly-HEME volume used per well/plate** |
|---|---|---|---|
| **Multiwell plates** | 96 well | 0.32 - 0.6 | 100 µl |
| | 24 well | 2 | 300 µl |
| | 6 well | 9.5 | 1 ml |
| **Dishes** | 35 mm | 8 | 1 ml |
| | 60 mm | 21 | 2 ml |
| | 100 mm | 56 | 2 -3 ml |

### 6.2.2. RNA isolation from cultured cells

After the appropriate treatment, RNA was extracted from the cells using 80% Trizol (Invitrogen), according to the manufacturer's protocol. Concisely, 0.2 ml of chloroform was added per 1 ml of Trizol and the samples were incubated on ice for 10 min before being centrifuged at 8000 rpm for 15 min at 4°C. The aqueous phase was then removed and transferred to a new tube. The RNA was precipitated out of solution with 0.5 ml isopropanol per 1 ml Trizol overnight at -20°C or at room-temperature for 10 min after vortexing. Samples were centrifuged at 8000 rpm for 30 min at 4°C and the RNA pellets washed with 75% ethanol. The RNA pellets were then air-dried before being re-suspended in 30 µl 0.01% diethylpyrocarbonate (DEPC)-treated $H_2O$. RNA was quantitated and 1-2 µg was electrophoresed on a 1.5% formaldehyde gel containing 0.5 µg/µl ethidium bromide to verify the integrity of the RNA.

### 6.2.3. Quantitative real-time RT-PCR analysis

Complementary DNA (cDNA) first strand synthesis, based on the protocol by Gelder *et al.* (1990)[233], was performed using 2 µg RNA, from either cultured cells or patient biopsies. This was performed in a final volume of 20 µl using 0.5 mM oligo-dT or random hexamer primers, 1 µl ImProm-II Reverse Transcriptase (Promega), 1 µl RNAsin (Promega), 2 mM $MgCl_2$, 1 mM dNTPs and 1X ImProm-II reaction buffer (Promega). First the primers were added to the RNA and were allowed to anneal at 70°C for 10 min, before the remaining components were added to the sample. The sample was then incubated at 42°C for 2 hours and 70°C for 10 min, to

inactivate the enzyme. The cDNA from both cultured cells and patient biopsies were aliquoted and stored at -80°C.

Real-time RT-PCR was performed to measure the levels of PAK3 mRNA in a sample, relative to an internal control or house-keeping gene, as described by Houghton and Cockerill (2006)[234]. This was done using 2 µl of cDNA with the StepOne Real-Time PCR System (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) and SYBR Green Fast qPCR Master Mix (KAPA Biosystems, Cape Town, SA), according to the manufacturer's instructions. One pair of primers, from the below table (Table 6.2), was used per sample. All primers were designed utilizing Primer-Blast (www.ncbi.nlm.nih.gov/tools/primer-blast) and the mRNA sequences were obtained from the NCBI website. Where possible, primers were designed to span exon/exon boundaries to exclude genomic DNA amplification. Primers were stored at -20° at 20 µM concentrations.

Samples were cycled at 95°C for 3 min, followed by 40 cycles of 95°C for 1 second and then the primer pair's annealing temperature (Ta) for 20 sec. All samples were performed in triplicate and the relative mRNA expression level of the gene of interest was calculated using the comparative threshold cycle ($C_T$) method[139]. Gene expression of PAK3 was standardised relative to an internal control: for rat expression, GAPDH, and for human, β-glucuronidase (GusB) or cyclophillin D. PAK3 levels in patient biopsy samples were normalised using both human internal controls - to account for the patient variability. The levels of GusB and cyclophillin D in each sample were averaged and this reading was used to normalize the PAK3 mRNA expression level. All PCR products were separated on a 2% agarose gel with ethidium bromide to ensure the amplification of a single product.

Table 6.2: Sequences of primers used for real-time RT-PCR analysis

| Primer | Sequence | PCR Product Size (bp) | Ta (°C) |
|---|---|---|---|
| rat PAK3 forward | 5'-TCACTCCTGAGCAAAGTAAACG-3' | | |
| rat PAK3 reverse | 5'-TCCCAGAGACCAGATATCAACTT-3' | 112 | 55 |
| human PAK3 forward | 5'-CCAGATCACTCCTGAGC-3' | | |
| human PAK3 reverse | 5'-CCAGATATCAACTTTCGGACC -3' | 109 | 60 |
| rat GAPDH forward | 5'-ATGACTCTACCCACGGCAAG-3' | | |
| rat GAPDH reverse | 5'-TACTCAGCACCAGCATCA-3' | 136 | 55 |
| human Gus B forward | 5'-CTCATTTGGAATTTTGCCGATT-3' | | |
| human Gus B reverse | 5'-CCAAGTGAAGATCCCCTTTTTA-3' | 81 | 55 |
| human cyclophilin D forward | 5'-TGAGACAGCAGATAGAGCCAAGC-3' | | |
| human cyclophilin D reverse | 5'-TCCCTGCCAATTTGACATCTTC-3' | 94 | 60 |

### 6.2.4. Harvesting and quantitating protein from cultured cells

After the necessary treatment, cells were lysed on ice using RIPA buffer (see solutions) containing 0.5 mM PMSF, 1X complete protease inhibitor cocktail (Roche, Basel, Switzerland), and 50 mM NaF, 2 mM $Na_3VO_4$ to inhibit the action of phosphatases. Cell lysates were sonicated before centrifugation at 10000 rpm for 10 min at 4°C to remove the cell debris. Protein samples were stored at -80°C before use. Protein concentrations were quantitated using the Bicinchoninic Acid (BCA) protein assay kit (Pierce) according to the manufacturer's protocol.

### 6.2.5. Western blot analysis

Protein was separated using the Mini Protean II System (Bio-Rad). 10-30 µg of protein was separated by 8% or 10% SDS-PAGE after 1X Laemmli loading dye (see solutions) was added to the samples and they were heat-denatured at 85 - 90°C for 2 min. One of two protein molecular weight makers was run concurrently with the samples to determine the size of separated

proteins: Kaleidoscope (BioRad) or Spectra Multicolour Braods Range Ladder (Fermentas). After electrophoresis, the proteins were transferred to a Hybond$^{TM}$-ECL$^{TM}$ nitrocellulose membrane (Amersham Life Sciences) using the wet/tank transfer system (BioRad). The membrane was subsequently blocked in 5% low fat milk in Tris-buffered saline with 0.1% Tween (TBST) for 1 hour at room-temperature and then incubated with the primary antibodies overnight with shaking. Post primary antibody incubation, membranes were washed three times in TBST for 10 min before the horseradish peroxidase-conjugated secondary antibodies were added for 1 hour at room temperature with shaking. (See Table 6.3 for optimised conditions and concentrations of the primary and secondary antibodies.) Excess secondary antibody was removed with three 10 min TBST washes and then the protein bands detected using the LumiGLO or LumiGLO reserve chemiluminescent substrate system (KPL Inc.). To re-probe membranes for another protein, antibodies were stripped from the membrane with 1 M glycine, pH 2.5, shaking for 14 min, flipping the blot over half way. The low pH was neutralised with 10% of the glycine volume of 1 M Tris pH 7.5. The membrane was rinsed in TBST before an additional 30 min of blocking prepared it to have an alternative primary antibody added. ImageJ software was used to quantitate protein expression levels, and bar graphs represent the expression of each protein relative to β-tubulin (or alternative loading control).

Table 6.3: Antibody concentrations and conditions

| **Primary Antibody** | **Primary Antibody Conditions** | **Secondary Antibody** | **Secondary Antibody Conditions** | **Substrate** |
|---|---|---|---|---|
| Anti-PAK3<br>sc-48826 Santa Cruz | 1:1000 in TBST | Goat anti-rabbit<br>#172-1019 BioRad | 1:5000 in 5% milk | LumiGLO |
| Anti-PAK3<br>sc-1871 Santa Cruz | 1:1000 in TBST | Donkey anti-goat<br>sc-2020 Santa Cruz | 1:5000 in 5% milk | LumiGLO |

| | | | | |
|---|---|---|---|---|
| Anti-PAK1<br>sc-882 Santa Cruz | 1:1000 in TBST | Goat anti-rabbit<br>#172-1019 BioRad | 1:5000 in 5% milk | LumiGLO |
| Anti-PAK2<br>sc-7117 Santa Cruz | 1:1000 in TBST | Donkey anti-goat<br>sc-2020 Santa Cruz | 1:5000 in 5% milk | LumiGLO |
| Anti-cJun (H79)<br>sc-1694 Santa Cruz<br>(N-terminus) | 1:1000 in 5% BSA | Goat anti-rabbit<br>#172-1019 BioRad | 1:5000 in 5% milk | LumiGLO |
| Anti-cJun<br>sc-44 Santa Cruz<br>(DNA binding domain) | 1:1000 in 5% BSA | Goat anti-rabbit<br>#172-1019 BioRad | 1:5000 in 5% milk | LumiGLO |
| Anti-p-cJun (Ser 63/73)<br>sc-16312 Santa Cruz | 1:1000 in 5% BSA | Goat anti-rabbit<br>#172-1019 BioRad | 1:5000 in 5% milk | LumiGLO<br>reserve |
| Anti-p-cJun (Ser63)<br>9261 Cell Signaling | 1:500 in 5% BSA | Goat anti-rabbit<br>#172-1019 BioRad | 1:5000 in TBST | LumiGLO<br>reserve |
| Anti-p-cJun (Ser73)<br>9164 Cell Signaling | 1:1000 in 5% BSA | Goat anti-rabbit<br>#172-1019 BioRad | 1:5000 in TBST | LumiGLO<br>reserve |
| Anti-ERK 2<br>sc-154 Santa Cruz | 1:1000 in 1% milk | Goat anti-rabbit<br>#172-1019 BioRad | 1:5000 in 5% milk | LumiGLO<br>reserve |
| Anti-pERK 1/2<br>(Thr202/Tyr204)<br>9106 Cell Signaling | 1:500 in 1% BSA | Goat anti-mouse<br>#170-6516 BioRad | 1:1000 in 2.5% milk | LumiGLO<br>reserve |
| Anti-JNK/SAPK<br>9258 Cell Signaling | 1:1000 in TBST | Goat anti-rabbit<br>#172-1019 BioRad | 1:5000 in 5% milk | LumiGLO |
| Anti-p-JNK 1/2<br>(Thr 183/185)<br>9251 Cell Signaling | 1:1000 in TBST | Goat anti-rabbit<br>#172-1019 BioRad | 1:5000 in 5% milk | LumiGLO<br>reserve |
| Anti-β-tubulin<br>sc-9104 Santa Cruz | 1:1000 in TBST | Goat anti-rabbit<br>#172-1019 BioRad | 1:5000 in 5% milk | LumiGLO |
| Anti-GAPDH<br>2118 Cell Signaling | 1:5000 in 5% milk | Goat anti-rabbit | 1:5000 in 5% milk | LumiGLO |

### 6.2.6. Extracting genomic rat DNA

Genomic rat DNA, as a template for the PCR amplification of the PAK3 promoter, was extracted from Rat1a-GFP cells. 2.6 x $10^6$ cells were plated in a 150 mm dish and grown until they were 80% in confluency. The cells were trypsinised, pelleted, washed and re-suspended in PBS for counting. The cells were then re-suspended in a cell-number-dependent volume of Digestion Buffer (see solutions): 0.3 ml for < 3 x $10^7$ cells or 1ml per $10^8$ cells. The sample was then shaken

overnight at 45°C. The following day, an equal volume of a phenol/chloroform/isoamyl alcohol mix was added. The sample was mixed and subsequently centrifuged at 10 000 rpm at 4°C for 10 min. The top aqueous phase was removed and precipitated with 20 µl 7.5 M ammonium acetate and 80 µl of absolute ethanol. To yield the precipitated DNA, the sample was centrifuged at 10 000 rpm for 2 min at 4°. The DNA pellet was washed with 70% ethanol, air dried and then re-suspended in 30 µl TE buffer pH 7.4. The DNA was left to dissolve at room temperature or 37°C overnight, before being stored at -20°C. The genomic DNA was then quantitated and the integrity of DNA confirmed on a 1% agarose gel ensuring a large molecular weight band (intact DNA) could be seen rather than a smear across a range of sizes (degraded DNA).

### 6.2.7. PCR Amplification of the PAK3 (-2436 to +149) promoter region

To investigate whether the PAK3 promoter was responsive to AP-1, the 5'-regulatory region of the rat PAK3 promoter (GenBank Accession Number: NC_005120) was cloned. Primers were designed to amplify a region approximately 2500 bp upstream and 100 bp downstream from the transcriptional start site. Restriction enzyme sites were added to the designed primers to facilitate the cloning of the promoter fragment into the reporter vector, $pGL_3$-Basic (Promega). These restriction sites chosen were confirmed to not be present in the sub-cloning vector, pGEM-T Easy (Promega), but were only present in the multiple cloning site of $pGL_3$-Basic. The following primers were used: F 5'-TG**ACGCGT**AGAGAAGGAAGCCAAGAATC-3' (Mlu1 site in bold) and R 5'-CGCTCGAGGTGTAAGACCCCAGACAGTT-3' (Xho1 site is underlined), and resulted in the amplification of the (-2436/+149) region of the rat PAK3 promoter.

The PCR was performed using 0.5 µM of each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 U of high fidelity Expand Plus DNA Polymerase (Roche), 1 X buffer and 500 ng of the rat genomic DNA. The PCR conditions were as follows: 95°C for 5 min; 30 cycles of 95°C for 30 seconds, 60°C for 30 seconds of annealing, 72°C for 3 min; with a final extension step of 72°C for 15 min. A gradient PCR was used to establish the optimal annealing temperature before the PCR was performed. The $MgCl_2$ concentrations were also optimised before the amplification PCR was performed.

### 6.2.8. Purification and A-tailing of the PAK3 (-2436 to +149) promoter region PCR product

The PCR products were electrophoresed on a 1% agarose gel and the size-confirmed product cut out and purified using the QiaQuick Gel Extraction Kit (Qiagen, Limburg, Netherlands) and the DNA pellet was re-suspended in 30 µl of the elution buffer. Half the volume of the amplified promoter fragment was then A-tailed using 5 U Taq Polymerase (Stratagene, La Jolla, CA, USA), 1 X Taq buffer, 2.5 mM $MgCl_2$ and 0.2 mM ATP at 70°C for 30 min. The A-tailing had to be performed additionally to the PCR reaction as a high fidelity polymerase used to minimize the errors incorporated in the PCR generates a blunt end, rather than an A-tail, as would be with the amplification by Taq polymerase.

### 6.2.9. Sub-cloning the PAK3 (-2436 to +149) promoter region into pGEM-T Easy

The amplified product was then sub-cloned into the pGEM-T Easy vector. This ligation was performed directly after the A-tailing reaction and comprised approximately 40 ng promoter fragment, 50 ng pGEM-T Easy, 1 X Rapid Ligation Buffer and 3 U T4 DNA Ligase (Promega). The

reaction was performed overnight at 4°C and confirmed through blue-white screening, where the inserted fragment disrupts the β-galactosidase gene of the pGEM-T Easy plasmid when transformed into JM109 competent cells (Promega) yielding white colonies. To transform the competent JM109 cells, half of the ligation mix was combined with 30 µl of JM109 cells and incubated on ice for 30 min. The mixture was then heat-shocked at 42°C for 2 min, before 0.45ml of LB was added and the mixture was incubated at 37°C for 1 hour. The cells were then plated onto LB agar plates containing 100 µg/ml ampicillin and previously spread with 100 µl 0.1 M IPTG and 20 µl 50 mg/ml Xgal. The plates were incubated overnight at 37°C to allow for the growth of the bacterial colonies.

**6.2.10. Small scale preparation and screening of pGEM-T Easy clones**

A number of white colonies were picked from the plates and individually inoculated into 5 ml LB containing 60 µg/ml ampicillin and grown overnight, shaking at 37°C. The following day, Rapid Plasmid Extractions were performed on all of the cultures after 500 µl of each sample was added to 500 µl 80% glycerol and stored at -80°C as a glycerol stock. The remaining culture was pelleted at 2000 rpm for 10 min at room temperature and re-suspended in 200 µl of Buffer P1 (Qiagen Maxiprep Kit, Qiagen) for 5 min at room temperature. 400 µl of Buffer P2 was then added and incubated on ice for 5 min, before 300 µl of Buffer P3 was added. The solution was mixed and incubated on ice for a further 5-10 min. 800 µl of the supernatant was then removed after two rounds of centrifugation at 13 000 rpm for 5 min in at room temperature, and 600 µl of isopropanol was added to precipitate the DNA at -20°C for 30 min. The DNA was pelleted at

13 000 rpm for 12 min at 4°C, washed twice in 70% ethanol, air dried and re-suspended in 50 µl of TE, pH 7.4.

The clones were then verified to contain the insert through restriction enzyme digestion with the MluI and XhoI sites cloned into the amplification primers, as well as two other restriction enzymes that cut within the insert, EcoRV and HindIII (data not shown).

### 6.2.11. Large scale preparation of the pGEM-T Easy clone and verification of the insert by sequencing

Before being cloned into the reporter vector $pGL_3$-Basic, the PAK3 (-2436 to +149) promoter region had to be verified by sequencing. Firstly, good quality plasmid DNA was prepared. The pGEM-T Easy plasmid containing the PAK3 promoter fragment was inoculated from the glycerol stock into 5 ml LB with 60 µg/ml ampicillin for 7 hours at 37°C. This 5 ml culture was then inoculated into 100 ml LB with 100 µg/ml ampicillin and grown overnight at 37°C. The following day the plasmid DNA was purified using the Qiagen MaxiPrep Kit (Qiagen) according to the manufacturer's instructions.

Since the promoter fragment was cloned into the pGEM-T Easy vector, the T7 (5'-TAATACGACTCACTATAGGG-3') and SP6 (5'-CGATTTAGGTGACACTATAG-3') primers, which bind either side of the pGEM-T Easy multiple cloning site, were used to sequence the inserted fragment. 500 ng of the prepared plasmid containing the PAK3 promoter fragment were combined with 6.4 pmol of either the T7 or SP6 primer, for two reactions sequencing from either side of the fragment. These sequencing reactions were performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's

protocol, with cycling conditions of 96°C for 1 min, followed by 25 cycles of 96°C for 10 seconds, 45°C for 5 seconds and 60°C for 4 min. The sequencing electrophoresis was performed by the University of Cape Town Human Genetics Sequencing Unit.

### 6.2.12. Preparation of promoter-luciferase constructs in $pGL_3$-Basic

Once the PAK3 (-2436 to +149) promoter region had been sequenced, it was excised from the pGEM-T Easy vector to be cloned into the luciferase reported vector, $pGL_3$-Basic. This excision was performed using the restriction enzyme sites, Mlu1 and Xho1, incorporated in the amplification primers. The $pGL_3$-Basic vector was also double digested with the Mlu1 and Xho1 enzymes in preparation for the insert and was gel purified along with the cut PAK3 promoter fragment from the pGEM-T Easy plasmid. The fragment and vector were then ligated together to form the $pGL_3$-Basic-pPAK3 (-2436/+149) plasmid, where the cloned PAK3 promoter fragment lies up stream of a promoter-less luciferase gene. The amount of PAK3 promoter fragment (insert) needed for the ligation was determined by the following equation:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}} = \text{ng of insert}$$

Where 100 ng of the vector was used and the insert:vector ratio was 6:1.

The calculated amount of digested insert was then combined with the digested vector and incubated at 45°C for 5 min, followed by 5 min on ice. 3 U of T4 DNA ligase (Promega), 1 X T4 DNA ligase buffer and 5% polyethylene glycol (PEG), to enhance the ligation, were then added to the mix and incubated at 16°C overnight. The following day, the whole ligation mix was transformed in JM109 competent cells and plated overnight. The next day, colonies were

picked and screened for the insert using the incorporated Mlu1 and Xho1 restriction enzyme sites, along with the two other sites which fall within the fragment, EcoRV and HindIII (Fig. 2.4). Once confirmed, the correct clone was grown up as described previously and purified using the Qiagen MaxiPrep Kit (Qiagen).

### 6.2.13. Luciferase promoter assays

3 x $10^4$ cells were seeded into 24-well plates and allowed to settle overnight. The following day, the media on the cells was changed to a volume of 0.5 ml and the transfection mix of 50 µl serum-free media, 10 ng of pRL-TK (Renilla luciferase) (Promega), 100 ng of $pGL_3$-basic or $pGL_3$-Basic-pPAK3 (-2436/+149) plasmid and 0.3 µl of TransFectin Lipid Reagent (BioRad) – where the Transfectin to plasmid ratio is 3:1 – was made up. pRL-TK was used as a control for transfection efficiency. The mix was left at room temperature for 15 – 20 min before being added to the cells. The cells were incubated with the transfection mix for 4 hours before the media on the cells was replaced with fresh complete media. In Rat1a-J4 cells, 2 µg/ml doxycycline was added at this stage to induce cJun/AP-1 expression. In Rat1a cells, cJun/AP-1 overexpression was achieved through the transfection of the pCMV-cJun plasmid. Cell lysates were prepared 48 hours after transfection using Passive Lysis Buffer (Promega) and luciferase activity measured using the Dual-Luciferase® Reporter Assay Kit (Promega) and measured on the Glomax 96 Microplate luminometer (Promega). Activity was normalised to the Renilla luciferase activity from pRL-TK in the same lysate.

In other promoter experiments, additional plasmids or siRNA were added to the transfection mix depending on experiment conditions. In these cases, the amount of TransFectin Lipid Reagent was increased accordingly, maintaining the 3:1 ratio of TransFectin to DNA.

### 6.2.14. Bioinformatic promoter analysis

The (-2436/+149) region of the PAK3 promoter was bioinformatically analyzed for putative AP-1 binding sites utilizing three programs: Conreal, MATCH and MatInspector. Conreal uses weight matrices of transcription factor binding sites to identify common transcription factor binding sites between orthologous sequences, while MATCH uses a library of positional weight matrices from TRANSFAC® Public 6.0 to predict transcription factor binding sites in a sequence. MatInspector identifies matches in the DNA sequences based on a large library of matrix descriptions for transcription factor binding sites, and assigns a quality rating to matches which allows for filtering and selection of certain matches. Each program revealed a number of alternative sites, locating both TRE and CRE consensus sites on the plus and minus strand. These sites are summarised in Fig. 2.6.

### 6.2.15. Generation of PAK3 promoter deletion constructs

Promoter deletion constructs of the (-2436/+149) PAK3 promoter were generated using restriction enzyme digestion – to release an unwanted region - and a re-ligation of the $pGL_3$-Basic-pPAK3 plasmid. In each digestion, the cloned Mlu1 site was utilised, along with restriction enzyme sites located within the cloned promoter (EcoRI for the -2364/+149 construct; PstI for the -684/+149 construct and NsiI for the -179/+149 construct). Once a region was excised, the

remaining linearised plasmid was gel purified and then the sticky ends were blunt-ended using T4 DNA Polymerase (Promega) before the plasmid was closed by self-ligation.

Each double digestion was performed using 50 µg plasmid – to ensure there was a sufficient amount of plasmid after the clean-up processes – together with 50 U of MluI and 50 U of the other required enzyme, the compatible buffer (Buffer D, Promega) and made up to a total reaction volume of 100 µl with $H_20$. The digestions were incubated overnight at 37°C. The next day, the digestion product was electrophoresed on a 1% agarose gel and the larger band (the plasmid minus the unwanted region) was excised and gel purified. All of the restriction enzymes chosen were sticky-end cutters, making them incompatible for relegation. Thus all linearised plasmids were then blunt-ended using T4 DNA polymerase which fills in the 5' overhangs and removes the 3' overhangs at 12°C, maintaining the fragment in frame. The reaction was set up using 10 U of the enzyme, 1 X T4 DNA Polymerase Buffer, 2 nM dNTPs, 2 mg/ml BSA and the whole volume of the gel purified DNA at 12°C for 20 min before deactivating the enzyme at 70°C for 10 min. The DNA was once again run on a 1% agarose gel and gel purified. The linearised plasmid was then blunt-end ligated after the DNA clumps were denatured at 45°C for 10 min and placed on ice for another 10 min. The ligation was performed using 10 µl of the blunt-ended plasmid and 2 µl T4 DNA Ligase with 1 X the T4 DNA Ligase buffer at 22°C overnight. The next day, the enzyme was denatured at 65°C for 10 min. The resultant plasmids were transformed into JM109 competent cells and the correct clones verified by restriction enzyme digestions. These plasmids were the transfected into Rat1a-J4 cells in the presence and absence of doxycycline for luciferase reporter assays (as described above).

### 6.2.16. Site-directed mutagenesis of the (+52/+60) putative cJun binding site

The (+52/+60) putative cJun binding site in the (+179/+149) PAK3 promoter construct was mutated using site-directed mutagenesis. The putative cJun binding site has the wildtype sequence of TGACGTCA. Primers were designed to incorporate the appropriate mutation which would specifically disrupt the transcription factor binding site but not any other site, and would not incorporate any additional sites into the promoter region. This site in the PAK3 promoter was mutated to ACGCGTTT (where the underlined regions highlight the mutated bases). Primer design and melting temperature calculation was performed using Primer X software (http://www.bioinformatics.org/primerx/) and resulted in the following primer set: F 5'-GGTACGGTGCAGAGCCCAGGacgCGTtaTAGCATAGAAGAGCTAGG-3' and R 5'-CCTAGCTCTTCTATGCTAtaACGcgtCCTGGGCTCTGCACCGTACC-3' where the mutated bases are in lowercase and the incorporated Mlu1 site is underlined. To perform the PCR, 50 ng of the template $pGL_3$-Basic-pPAK3 (+179/+149) plasmid was combined with, 0.2 nM of the forward primer, 0.2 nM of the reverse primer, along with 0.2 mM dNTPs, 2.5 U ExpandPLUS high fidelity DNA Polymerase and 1 X Polymerase Reaction Buffer. PCR cycling conditions were: 95°C for 30 seconds, followed by 18 cycles of 95°C for 30 seconds, 55°C for 1 minute and 72°C for 6 minutes, and a final extension step of 72°C for 20 min.

After PCR amplification, Dpn1 (Promega) was used to digest the template plasmid at 37°C for 2-3 hours before the products were transformed into JM109 highly competent cells (Promega). The introduced novel Mlu1 restriction enzyme site was used to confirm the inclusion of the mutation.

### 6.2.17. Preparation of nuclear protein for the Electrophoretic Mobility Shift Assay (EMSA)

2.6 x $10^6$ Rat1a-J4 cells were plated in two 150 mm culture dishes and allowed to settle overnight. The following day, one plate was treated with 2 µg/ml doxycycline to induce cJun/AP-1 over-expression. 48 hours later, the nuclear protein was extracted from both conditions. To achieve this, cells were washed and harvested in PBS through scraping and the lysate was centrifuged at 1000 rpm for 5 min at 4°C. The cell pellets were then re-suspended in the equal volume – to the pellet size - of Buffer A (see solutions). This mixture was incubated on ice for 15 min. The cells were then manually lysed using a 1 ml syringe with a 26-gauge needle performing 5 cycles of slow aspiration and ejection. The resultant lysate was then centrifuged at 12 000 rpm at 4°C for 1 min and the pellet re-suspended in Buffer C (see solutions), a volume equivalent to two-thirds of the pellet size. This mixture was then shaken at 4°C for 30 min. The supernatant was then collected after centrifugation at 12 000 rpm for 5 min at 4°C and dialyzed for 2 hours against Buffer D (see solutions). The resultant nuclear protein was then stored at -80°C

### 6.2.18. Electrophoretic Mobility Shift Assay (EMSA)

<u>Oligomer design</u>

Two oligomers (20-45mers), one containing the wildtype (+52/+60) PAK3 promoter region and another with a mutated version of the putative AP-1 binding site, were required for the assay. The wildtype oligomer was formed utilizing two single stranded oligomers (sense: 5'-CCCAGG**TGACGTCA**TAGCAT-3' and antisense: 5'-ATGCTA**TGACGTCA**CCTGGG-3', where the (+52/+60) site is in bold), while the mutant oligomer was formed using the forward and reverse

primers from site directed mutagenesis (as mentioned above). The site directed mutagenesis primers could be used as they were complimentary to one another. The single stranded oligomers of both the wildtype site and the mutant site were then annealed together to form the double stranded oligomers.

Annealing single stranded oligomers

Equal concentrations (approximately 10 µg) of the sense and antisense oligomers were combined and the total volume made up to 30 µl with annealing buffer (see solutions). The oligomer mixtures were placed at 95°C for 3 min, 65°C for 10 min, 37°C for 1 hour and were then cooled at room temperature. Each double stranded oligomer was run on a 2% agarose gel to confirm the annealing. The product was then gel purified and quantitated.

Labeling oligomers

75 µg of each oligomer was added to 2 µl of 4 x $10^4$ cpm/µl γ-$^{32}$P-ATP along with 1 µl of T4 PNK and T4 buffer in a volume of 20 µl. The mixture was incubated at 37°C for 30 min, after which the reaction was stopped with the addition of 2 µl of 0.5 M EDTA. The mixture was then loaded onto a Sephadex G50 column (Sigma-Aldrich) and the purified probe was collected after centrifugation at 3000 rpm for 2 min. The labeling was confirmed by checking the activity with the scintillation counter.

Forming the protein/DNA complexes

3-8 µg of crude nuclear protein, extracted from Rat1a-J4 cells, per condition was incubated at room temperature for 15 min with 4 µg of poly(dI/dC) and 5 X Incubation Buffer. Following this,

the unlabeled wildtype of mutant competitor oligomers in a 75 X molar excess, or the supershift analysis antibodies were added to the appropriate samples, after which, the mixture was incubated on ice for 20 min. Protein-DNA complexes were formed when γ-$^{32}$P-labled oligomers were added and the mixtures incubated for a further 30 min on ice. 2 µl of bromophenol blue was then added to the 20 µl sample volume. The protein-DNA complexes were then separated on a non-denaturing 5% polyacrylamide gel at 150V for approximately 2 hrs at 4°C in 1xTBE. After electrophoresis, the gel was dried and exposed to X-ray film overnight. For supershift analysis, anti-cJun (sc-1694 and sc-44 (Santa Cruz)), anti-JunB (sc-73 (Santa Cruz)) and anti-JunD (sc-74 (Santa Curz) antibodies were used.

**6.2.19. Chromatin Immunoprecipitation (ChIP) Assay**

Four 150 mm culture dishes were plated with 2.6 x $10^6$ Rat1a-J4 cells and allowed to settle overnight. The following day, two of the four dishes were treated with 2 µg/ml doxycycline. 48 hours later, protein/DNA complexes from untreated and doxycycline-induced Rat1a-J4 cells were cross-linked with 1% formaldehyde for 10 minutes at room temperature. The reaction was then neutralised with 0.125 M glycine pH 2.5 for 5 min. Cells were then washed twice in PBS, pelleted at 200 x g for 5 min at 4°C and re-suspended in lysis buffer (see solutions). After a 10 min incubation on ice, the solution was sonicated to sheer chromatin to between 400-1000-bps in length. The cell debris was then pelleted at 15 000 rpm at 4°C for 10 min, and 20 µl of each sample was set aside as the input fraction. The remaining solution was diluted 1:10 with dilution buffer and pre-cleared with blocked protein-A agarose beads (Merck) for 2 hours at 4°C after which the beads were removed through centrifugation at 15 000 rpm at 4°C for 1 min. The

protein-A beads were blocked overnight at 4°C using a solution of lysis buffer:dilution buffer at a 1:10 ratio containing 100 µg/ml salmon sperm DNA and 5% BSA, after which the beads were washed in PBS and re-suspended in 1:10 lysis buffer:dilution buffer solution. The cleared chromatin was then either immunoprecipitated with or without 20 µl of anti-cJun antibody (sc-44 Santa Cruz) overnight at 4°C. The following day, 45 µl of pre-blocked protein-A agarose beads (Merck) were added for 2 hours. Bead-bound complexes were recovered by centrifugation and washed sequentially with TSE I, TSE II, Buffer III and TE, pH 7.4. Bound chromatin was released from beads using elution buffer and DNA released from formaldehyde cross-links by heating at 65°C overnight. DNA was purified with the Wizard SV Gel and PCR Clean-Up System (Promega) as per the manufacturer's instructions. RT-PCR was performed on the purified chromatin using primers spanning the (+52/+60) PAK3 promoter site (FWD: 5' AGATTGGTCCCCAGTAGCCC 3' and REV: 5' ACCCCAGACAGTTTGCGG 3') using the StepOne Real Time PCR System (Applied Biosystems) and SYBR qPCR Universal Kit (KAPA Biosystems) with the cycling conditions of: 95°C for 3 min, followed by 40 cycles of 95°C for 1 second and 55°C for 20 seconds.

### 6.2.20. Transfection of cells with siRNA, shRNA and plasmids

In order to determine the role that PAK3 plays within transformed cells lines, siRNA and shRNA targeted against PAK3 was used to reduce the levels of PAK3 protein so that we could assay the biological effect of the loss. With each siRNA/shRNA and plasmid transfection, the equivalent of $1 \times 10^5$ cells were plated in a 35 mm dish and allowed to settle overnight. The following day, with the cells at approximately 60% confluency, the media on the dish was changed to 1 ml.

The transfection mix was made up in 50 µl serum-free media with a 3:1 ratio of TransFectin Lipid Reagent (BioRad) to siRNA or 100 µg of plasmid according to the manufactures protocol. The mix was agitated and incubated at room temperature for 15 min, before being added dropwise to the plate of cells. The cells were incubated at 37°C for 4-6 hours with the transfection mix, after which the medium was replaced with fresh full media. The effect of each transfection was monitored by western blot analysis.

### 6.2.21. Cell proliferation assay (MTT assay)

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) (Sigma-Aldrich) assays were performed to measure the viability of cells. Proliferating cells with intact mitochondria can metabolise MTT, a yellow tetrazolium salt, into purple formazan crystals. After dissolving these crystals, the reading at OD595 is thought to be proportional to the number of viable cells. These readings, taken over time, represent the proliferation of cells.

Depending of the cell size, 3 – 6 x $10^3$ cells, pre-transfected with siRNA or shRNA and treated with doxycycline in the case of Rat1a-J4 cells, were seeded into 96-well plates in 100 µl media. Each day thereafter, 10 µl MTT reagent was added to each well. Four hours later, 100 µl solubilization solution was added to each well and incubated overnight at 37°C to dissolve the formed crystals. The following day, the absorbance was read at OD595 using a BioTek microplate reader (BioTek, Winooski, VT, USA).

To measure anchorage-independent proliferation, the 96-well plates were coated with Poly-HEME prior to plating and cells were plated in 150 µl 1% methylcellulose. The MTT was added as with anchorage-dependent assays, however, before solubilization solution was added, 100 µl

from each well was removed and placed in another 96-well plate. It was found that the solubilization solution not only dissolved the crystals, but also the Poly-HEME that coated the wells. This skewed the readings of the assay. All MTT assays were performed using six wells per condition and repeated at least two times. The readings were normalised by subtracting the background reading of the media only wells.

### 6.2.22. Phase contrast microscopy

Phase contrast microscopy was used to capture the morphological changes associated with transformation and the effect of PAK3 inhibition in Rat1a-J4 and SVWI38 fibroblasts. Rat1a-J4 cells were plated onto sterilised cover slips in 35mm dishes, and allowed to settle overnight under adherent growth conditions. The rat fibroblast cell line was then transfected with control or PAK3 siRNA and additionally treated with 2 µg/ml doxycycline to induced cJun/AP-1 expression. 72 hours after transfection and doxycycline treatment, the Rat1a-J4 cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature and mounted on slides. Cells were viewed under visual light and phase contrast pictures were taken using a standard fluorescence microscope. Transformed human fibroblasts, SVWI38, were transfected with control or PAK3 siRNA and along with their normal counterparts, WI38, were photographed live using the Motic MotiCam digital microscope camera.

### 6.2.23. Actin staining

Cells were prepared similarly to that for phase contrast microscopy. After fixing, cells were washed twice in 0.04% PBS-Tween and blocked in 1% BSA for 30 min. Actin was labeled with 50

µg/ml Phalloidin-Tetramethylrodamine B isothiocyanate (Sigma-Aldrich) in 15% BSA for 30 min at room temperature. After washing twice with PBS, the nuclei of the cells were stained with 100 ng/ml DAPI in PBS for 10 min at room temperature. The stained cells were then washed twice in PBS and mounted on slides with Mowiol. Phalloidin images were viewed using the Zeiss LSM 510 Meta microscope with a Mai Tai two photon laser and captured using the ZEN 2009 software. AxioVision 4.7 software was used to measure changes in cell area, while the number of cytoplasmic protrusions per cell was counted by eye and averaged for each condition.

### 6.2.24. Motility/migration assays

Cell migration assays were performed using 12-well-plate Transwell migration chambers (Costar, Cambridge, MA, USA) with an 8 µm pores. Cells were pre-transfected with either control or PAK3 siRNA/shRNA and 24 hours later the cells were serum starved in 1% serum-containing media, before the motility assay was set-up the next day (48 hours after transfection). In the case of Rat1a-J4 cells, the cells were transfected with siRNA and treated with doxycycline for 2 ½ days (just short of 72 hours) before the transwell was set up. The chambers were placed into 12-well plates containing 1ml of 15% FCS-containing media, where the high serum content acts as a chemoattractant for the cells. Depending of the cell size, 3-6 x $10^4$ cells were then plated in 0.5 ml of 1% FCS-containing media onto the porous membrane of the inserted chamber. The cells were incubated at 37°C and allowed to migrate through the membrane to the underside of the insert for 24 hours. After the incubation, the cells on top of the membrane, which had not migrated, were removed with a cotton swab. However, motile cells which had moved to the underside of the membrane were fixed in 1 ml of methanol for 5

min and stained with 1 ml of crystal violet (0.2% w/v in 2% methanol) for 5 min. The inserts were then rinsed multiple times, using cotton swabs to ensure the sides and edges of the inserts were cleaned properly. Along with every condition, performed in triplicate, a fourth chamber of cells were plated to serve as a cell number plating control between the conditions. The cells on the top of this chamber were not removed with a cotton swab, instead the cells on the top and bottom of the membrane were stained and served as a total cell count to which the number of migrated cells could be normailsed for each condition. The stained membranes were left to dry overnight at room temperature before being scanned and quantified using ImageJ software. As an alternative quantitation, the stained cells were then solubilised with 50% acetic acid and the absorbance read at OD595. Each condition was performed in triplicate and repeated.

**6.2.25. Puromycin cell viability assays**

Cell sensitivity to puromycin, as a selection marker for the transfection with the PAK3 shRNA related plasmids, was tested by treating both HeLa and A224 cells with varying concentrations of the drug. 1 x $10^5$ cells were plates into 6-well dishes and left over-night at 37°C to settle before being treated with the following concentrations of puromycin: 0, 0.5, 1.0, 1.5, 2.0, 2.5 µg/ml. Images of the cells were taken after 48 and 72 hours of incubation with the drug. A concentration capable of killing the whole dish of cells within 48 hours was picked for each cell line with which to select for the transfection of the shRNA plasmids.

### 6.2.26. Transient and stable transfection with PAK3 shRNA

To prepare stable PAK3 knock-down clones or to transfect cells transiently with the shRNA, 5 x $10^5$ cells were plated into 6-well plates. The following day, the media was changed to 1 ml and the transfection mix was made up with serum-free media, Transfectin Lipid Reagent (BioRad) and 1 μg/ml of the empty/scrambled/PAK3 shRNA vector. 4 hours after transfection, the media was replaced on the cells. For the stable clones, the cells were given 24 hours to recover after transfection before the selection marker, puromycin, was added. The media containing the selection marker was replaced every 3–4 days. Four individual pools of PAK3 shRNA transfected clones were selected and western blot analysis was used to confirm the clones with the greatest reduction in PAK3 protein level, compared to the empty or scrambled vector clone. For the transiently transfected cells (those cells that could not successfully be made into a stable cell line), no selection marker was used and protein was harvested 48 hours after transfection and western blots performed to show the transient reduction in PAK3 protein level.

### 6.2.27. Sequencing CA plasmid

In order to investigate the potential feedback–loop between PAK3 and AP-1, we acquired the pCMV-myc-CA-PAK3 plasmid, containing the mouse PAK3 cDNA with an activating mutation: T421E. In order to confirm the presence of this mutation sequencing was performed using the following primers: FWD 5'-TGACAGCTTGGATAACGA-3', REV 5'-ACGGCTACTGTTCTTAAT-3' and FWD 5'-TTGCACCAAGACCAGAGCAT-3, REV 5'-TTGGGGTTCTTATTTTCCCTCA-3'. 500 ng of the plasmid was combined with 6.4 pmol of either the forward or reverse primer and the sequencing reactions performed using the BigDye Terminator v3.1 Cycle Sequencing Kit

(Applied Biosystems) according to the manufacturer's protocol, with the cycling conditions of 96°C for 1 min, followed by 25 cycles of 96°C for 10 seconds, 45°C for 5 seconds and 60°C for 4 min. The sequencing electrophoresis was performed by the UCT Human Genetics Sequencing Unit.

### 6.2.28. Transfection with PAK3 CA expressing plasmid

To over-express constitutively-activated (CA) PAK3 in SVWI38 and HeLa cells, the equivalent of 1 x $10^6$ cells were plated in a 100 mm dish. The following day, the media on the cells was changed to 6.5 ml of full media, and the transfection mix was made up with 338 µl serum-free DMEM, 6 µl Transfectin Lipid Reagent (BioRad) and 2 µg of the plasmid, which was incubated for 15-20 min at room temperature before being added to the cells. After 4 hours of incubation with the transfection mix, the media on the cells was replaced. 48 hours later, protein was harvested from the cells for western blot analysis.

### 6.2.29. AP-1 Luciferase reporter assays in response to CA PAK3 over-expression

To test whether over-expression of CA PAK3 resulted in an increase in AP-1 activity, the 4XAP-1-luc (described previously) plasmid was transfected into SVWI38 and HeLa cells, along with the pCMV and pCMV-myc-CA-PAK3 plasmids. The pRL-TK (Promega) plasmid was co-transfected to control for transfection efficiency. 12 x $10^4$ cells were plated in 35 mm dishes and left to settle overnight. The following day, the media was changed to 1 ml on the cells and the transfection mix was made up in 60 µl of serum-free DMEM with 100 ng of the 4xAP-1-luc plasmid, 100 ng of either the pCMV or pCMV-myc-CA-PAK3 plasmid, and 20 ng of the Renilla (pRL-TK) plasmid

together with a 3:1 ratio of DNA: TransFectin Lipid Reagent. 4 hours later, the transfection mix was removed from the cells and fresh full media added. Cell lysates were prepared after 48 hours using Passive Lysis Buffer (Promega). In the promoter experiments utilizing TPA and SP600125, the timing varied slightly. Cells were plated and transfected as before, however, when TPA was used to induce AP-1 activity, pCMV-myc-CA-PAK3 was not included in the transfection. When the media was changed 4 hours after transfection, 40 μM SP600125 was added to the appropriate condition. 16 hours later, 100 nM TPA was added to the required conditions to induced AP-1 activity for 4 hours, before the cells were harvested 24 hours after transfection. The luciferase activity was measured using the Dual-Luciferase® Reporter Assay Kit (Promega) and measured on the Glomax 96 Microplate luminometer (Promega).

**6.2.31. Co-immunoprecipitation assay**

To investigate whether cJun and PAK3 directly interact with one another, both the cJun and PAK3 proteins were over-expressed, using the pCMV-cJun and pCMV-myc-CA PAK3 plasmids respectively, in SVWI38 and HeLa cells. Following this, a PAK3 specific antibody, conjugated to a sepharose beads (Calbiochem), was used to pull-down all PAK3 protein and related complexes. This protein lysate was then subjected to western blot analysis for cJun.

Preparing the protein lysate

Two 100 mm dishes were plated with 1 x $10^6$ cells and were left overnight. The following day, both dishes were transfected with the pCMV-cJun (150 ng/ml) and pCMV-myc-CA PAK3 (300ng/ml) plasmids, and the media changed 4 hours later. After 48 hours, the protein from these cells was harvested in a non-denaturing buffer to ensure the protein/protein complexes

were not denatured. Once extracted in the buffer, the lysate was vortexed, incubated on ice for 15 min, sonicated twice for 10 seconds, spun down at 10 000 x g for 20 min at 4°C to remove the cell debris and quantitated using the BCA protein assay kit (Pierce). 1 mg of protein was needed: 500 µg for the pull-down experiment and 500 µg for the no antibody control.

Cross-linking antibody to Sepharose Beads

In order to make the pull-down more effective, the PAK3 antibody was cross-linked to the sepharose beads. Sepharose G beads were used as they are more specific for goat antibodies, as opposed to A beads which are better for rabbit-raised antibodies. 50 µl of Sepharose beads were washed twice and re-suspended in 100 µl PBS with 1 X protease inhibitors (Roche), before 2 µg of the PAK3 antibody (sc-1871 Santa Cruz) was added. The antibody and beads were rotated at room temperature for 1 hour, followed by the addition of the crosslinking agent Suberic acid bis(N-hydroxysuccinimide ester) (DSS) (Sigma-Aldrich) to a final concentration of 650 µg/ml. The mixture was rotated for a further hour at room temperature. The cross-linked beads were then washed three time in PBS, three times in 0.1M glycine pH 2.5 and twice in PBS.

Preclearing of the protein lysate

100 µl of Sepharose G beads (not those cross-linked to the antibody) were washed twice and re-suspended in 100 µl PBS, before being added to the 1 mg of protein lysate in a 2 ml volume. The protein was cleared by rotating the beads and protein for 1 hour at 4°C. The beads were then pelleted and discarded after centrifugation at 13 000 rpm for 2 min at 4°C. The protein was now ready to be used in the assay.

Immunopreciptitation

The pre-cleared protein was divided into two 500 µg lots. To one lot, 50 µl of the crosslinked antibody was added (pull-down experiment) and to the other, 50 µl of beads washed twice in PBS were added (no antibody control). These two conditions were incubated rotating overnight at 4°C. The following day, the beads were pelleted by centrifugation at 13 000 rpm for 2 min at 4°C and washed three times in PBS with protease inhibitors. The bead pellets were each re-suspended in 25 µl of 2 X loading buffer and the cross-links denatured at 95-100°C for 5 min, releasing the protein from the beads. The beads were then discarded through centrifugation, and 10 µl of each protein lysate loaded on an SDS-PAGE gel for electrophoresis and western blot analysis to detect cJun and PAK3 protein. These two conditions were run alongside a sample of the original input lysate (before pre-clearing) to act as a positive control for the expression of both proteins.

**6.2.32. Statistical analysis**

All experiments were performed in triplicate and were represented as the mean $\pm$ standard error of the mean. A Student's *t*-test, performed using either Microsoft Excel or Graphpad Prism, was used to compare groups of samples and a p-value of less than 0.05 was considered statistically significant. P-values $\leq 0.05$ were marked with a (*), while p-values $\leq 0.01$ were marked with (**).

## 6.3. SOLUTIONS

### 6.3.4. Tissue culture solutions

PBS

137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_20$ (pH7.4), 1.4 mM $KH_2PO_4$

Autoclave the solution.

Trypsin-EDTA

0.05% trypsin, 137 mM NaCl, 2.7 mM KCl, 4.8 mM $Na_2HPO_4.2H_2O$, 1.4 mM $KH_2PO_4$, 10 mM EDTA (pH 8.0)

16% Paraformaldehyde (PFA)

Dissolve 16 g Paraformaldehyde in 80 ml $dH_20$ by stirring at 60°C for 1 hour, ensuring the temperature does not exceed 60°C. Add drops of 10 M NaOH until the solution clears and pH to 7. Make the solution up to 100 ml and filter-sterilize using a 0.45 µm filter. Store at -20°C.

Cell-freezing media

70% DMEM, 20% FCS, 10% DMSO

MTT (5mg/ml)

Dissolve 100 mg MTT in 20 ml PBS by vortexing the solution and incubating it at 37°C for 15 min. Filter-sterilize using a 0.2 µm filter. Protect from the light and store at 4°C for up to one month.

Solubilization Reagent

Dissolve 25 g SDS in 250 ml $dH_20$. Add 76.6 µl concentrated HCl.

Blasicidin (5 mg/ml)

Dissolve powder in $H_2O$ and filter sterilize. The pH of final solution should not exceed pH 7. Aliquot and store at 4°C for 1-2 weeks, -20°C for 6-8 weeks and -80°C for a longer period. Upon thawing, discard remainder of the aliquot.

### 6.3.1 RNA solutions

10X MOPS Buffer

0.2 M MOPS, 0.05 M NaAcetate, 0.01 M EDTA

Autoclave the solution.

RNA loading buffer

Combine 7.2 ml Formamide, 1.6 ml 10X MOPS, 2.6 ml 37% Formaldehyde, 1.8 ml $dH_20$, 0.8 ml Glycerol and 0.8 ml 0.25% Bromophenol Blue. Mix well before use.

1.5% agarose gel containing formaldehyde

Dissolve 0.75 g Agarose in 5 ml 10X MOPS and 42 ml $dH_20$ by heating. As the solution is cooling, add 2.7 ml 37% Formaldehyde and 2.5 µl Ethidium Bromide. Cast the gel and allow the agarose to set.

DEPC-treated water

Add 0.01% DEPC to $dH_20$ and leave overnight in a fumehood. Autoclave the solution the following day.

75% Ethanol

Make from 96% Ethanol, as absolute Ethanol may contain benzene.

### 6.3.2 Protein solutions

RIPA buffer

150 mM NaCl, 1% Triton X-100, 1% NaDeoxycholate, 0.1% SDS, 10 mM Tris (pH 7.2)

4% Stacking Gel

Combine 3.65 ml $dH_20$, 0.625 ml 1 M Tris (pH 6.8), 0.65 ml 30% Acrylamide/bis-Acrylamide and 50 µl 10% SDS. Add 60 µl 10% Ammonium Persulphate and 6 µl TEMED to polymerise the gel.

10% Separating Gel

Combine 2.75 ml $dH_20$, 3.75 ml 1 M Tris (pH 8.8), 3.35 ml 30% Acrylamide/bis-Acrylamide and 100 µl 10% SDS. Add 200 µl 10% Ammonium Persulphate and 20 µl TEMED to polymerise the gel.

4X Laemmli Loading Dye

250 mM Trisl (pH 6.8), 6% SDS, 0.005% Bromophenol Blue, 40% Glycerol, 10% β-mercaptoethanol

10X Running Buffer

Dissolve 40 g Glycine, 63.2 g Tris and 10 g SDS in 1000 ml $dH_20$.

10X Transfer Buffer

Dissolve 144 g Glycine and 38 g Tris in 1000 ml $dH_20$.

1X Transfer Buffer

Dilute 100 ml 10X Transfer Buffer with 700 ml $dH_20$ and add 200 ml methanol or Isopropanol.

Normal Coomassie Staining Solution

50% Methanol, 10% Acetic Acid, 0.05% Coomassie Brilliant Blue

Normal Destain

5% Methanol, 7% Acetic Acid

TBST

50 mM Tris (pH 7.5), 150 mM NaCl, 0.1 % Tween-20

### 6.3.3 DNA solutions

Digestion buffer for genomic DNA isolation

100 mM NaCl, 10 mM Tris (pH 8), 25 mM EDTA (pH 8), 0.5% SDS, 0.1 mg/ml Proteinase K

TE Buffer

10 mM Tris (pH 8), 1 mM EDTA (pH 8)

Autoclave the solution.

TBE

89 mM Tris, 89 mM Boric Acid, 2 mM EDTA

Autoclave the solution.

### 6.3.5. Bacterial solutions

Luria Broth (LB) medium

Dissolve 10 g Tryptone, 5 g Yeast Extract and 10 g NaCl in 1000 ml $dH_20$. Add 1 mM NaOH and autoclave the solution.

LB agar

Dissolve 10 g Tryptone, 5 g Yeast Extract, 10 g NaCl and 15 g Agar in 1000 ml $dH_20$. Add 1 mM NaOH and autoclave the solution. When slightly cooled, add selection marker (100 ug/ml Ampicillin) before pouring the molten solution into bacterial dishes.

Ampicillin (10 mg/ml)

Dissolve 100 mg Ampicillin in 10 ml $dH_20$. Filter-sterilize and store at -20°C.

X-gal (50 mg/ml)

Dissolve 50 mg X-gal in 1 ml DMSO. Protect from the light and store at -20°C.

IPTG (0.1 M)

Dissolve 72 mg IPTG in 3 ml $dH_20$. Filter-sterilize and store at 4°C.

### 6.3.6. EMSA solutions

Buffer A

10 mM HEPES (pH 8), 1.5 mM $MgCl_2$,10 mM KCl, 1 mM DTT, 1X Complete Protease Inhibitor (Roche)

Buffer C

20 mM HEPES (pH 8), 1.5 mM $MgCl_2$, 25% Glycerol, 420 mM NaCl, 0.2 mM EDTA (pH 8), 1 mM DTT, 1X Complete Protease Inhibitor (Roche)

Buffer D

20 mM HEPES (pH 8), 20% Glycerol, 100 mM KCl, 0.2 mM EDTA, 1 mM DTT, 1 ug/ml Pepstatin, 1 ug/ul Leupeptin

Store at 4°C.

Annealing Buffer

100 mM K acetate, 30 mM HEPES (pH 7.4), 2 mM Mg acetate

5X Incubation Buffer (IB)

100 mM HEPES (pH 7.9), 250 mM KCl, 1 mM EDTA, 5 mM $MgCl_2$, 20% Ficoll 400, 2.5 mM DTT

5% Non-denaturing gel

Combine 6.25 ml 40% Acrylamide, 2.5 ml 10X TBE, 40.25 ml $H_20$. Add 1 ml APS and 25 µl TEMED to polymerise the gel.

### 6.3.7. ChIP solutions

Lysis Buffer

1% SDS, 5 mM EDTA, 50 mM Tris (pH 8.1), 1X Complete Protease Inhibitor (Roche), 1 mM PMSF

Dilution Buffer

1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris (pH 8.1), 1X Complete Protease Inhibitor (Roche), 1 mM PMSF

TSEI

0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris (pH 8.1), 150 mM NaCl

TSEII

0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris (pH 8.1), 500 mM NaCl

Buffer III

0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris (pH 8.1)

Elution Buffer

1% SDS, 0.1 M $NaHCO_3$

### 6.3.8. Anchorage independent growth

Polyheme (12 mg/ml)

Dissolve 2.4 g Poly-2-hydroxyethyl Methacrylate (Polyheme) in 20 ml 96% Ethanol in a water bath at 65°C for 1-2 hours. Dilute 1:10 in 96% Ethanol.

Methylcellulose (1%)

Autoclave 1 g Methylcellulose powder in a glass bottle. Warm 100 ml cell culture media and add to powder. Shake overnight at 4°C to dissolve.

### 6.3.9. Co-immunoprecipitation

Non-denaturing buffer for protein harvest

1 % Triton X-100, 120mM NaCl, 1mM $CaCl_2$, 25mM Tris (pH 7.4), 1 X Complete Protease Inhibitor (Roche)

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